Entwicklung von Verfahren für die spezifische, serologische Diagnostik von Dengue- und Zika-Virusinfektionen mit modifizierten *Envelope* Proteinen

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Abkürzungsverzeichnis

ADE	Antikörper vermittelte Infektionsverstärkung (*antibody dependent enhancement*)
DENV	Dengue-Virus
DHF	Dengue-hämorrhagisches Fieber
DSS	Dengue-Schock Syndrom
E-Protein	*Envelope* Protein
FL	Fusionsschleife (*fusion loop*)
FSME bzw. TBEV	Frühsommer Meningoenzephalitis Virus
JEV	Japanische-Enzephalitis-Virus
NASBA	*nucleic acid sequence based amplification*
NS1	Nichtstrukturprotein 1
prM/M Protein	*precursor* Membran/Membran Protein
VLP	*Virus like particle*
WNV	West-Nil-Virus
wt	Wildtyp
YFV	Gelbfieber-Virus
ZIKV	Zika-Virus

1 Einleitung

1.1 Einführung und Struktur der Arbeit

Das Dengue-Virus (DENV) ist ein in tropischen und subtropischen Regionen endemisches, humanpathogenes Virus, welches jährlich 390 Millionen Infektionen verursacht. Von diesen sind etwa 95 Millionen klinisch manifestiert [1]. DENV wird aufgrund der Antikörper-vermittelten Immunantwort in vier co-zirkulierende Hauptserotypen (DENV 1-4) aufgeteilt, wobei nach einer Primärinfektion eine lebenslange Immunität nur gegen den homologen Serotyp vorliegt. Die Sekundärinfektion mit einem heterologen Serotyp erhöht die Wahrscheinlichkeit einer erschwerten, lebensbedrohlichen Symptomatik wie die des hämorrhagischen Fiebers oder des Dengue-Schock Syndroms [2]. Eine präzise und schnelle Diagnostik spielt daher sowohl bei der symptomatischen Behandlung, als auch bei der epidemiologischen Überwachung von Dengue eine entscheidende Rolle. Jedoch erschwert die hohe Kreuzreaktivität von Antikörpern flaviviraler Infektionen vor allem die serologische Diagnostik [3]. Weiterhin wird seit der starken Ausbreitung des Zika-Virus (ZIKV) in DENV-endemischen Gebieten Südamerikas, eine massive Co-Zirkulation beider Viren beobachtet. Die ähnliche Symptomatik, wie z. B. Fieber, Myalgie und Exantheme, erfordert eine labordiagnostische Differenzierung von DENV und ZIKV, welche jedoch wegen der hohen strukturellen Homologie zu umfassenden Kreuzreaktivitäten in serologischen Tests führt [4,5].

Diese Arbeit befasst sich mit der Entwicklung einer Methode für die spezifische serologische Detektion von DENV- und ZIKV-Infektionen auf der Basis von modifizierten *Envelope* Proteinen. Die Dissertation ist als kumulative Publikationspromotion strukturiert und besteht aus zwei Publikationen mit Erstautorenschaft in peer-review Zeitschriften. Diese enthalten den Methoden- und Ergebnisteil, sowie eine detaillierte Diskussion der Ergebnisse.

1.2 Dengue- und Zika-Virus: Struktur und Taxonomie

Dengue (DENV) und Zika (ZIKV) sind Vektor-übertragene RNA-Viren, welche taxonomisch zur Familie der *Flaviviridae* im Genus *Flavivirus* zugeordnet werden. Diese umfasst über 70 Spezies, zu denen weitere bedeutende human- und tierpathogene Viren wie das West-Nil-Virus (WNV), das Gelbfieber-Virus (YFV), das Frühsommer-Meningoenzephalitis-Virus (FSME bzw. TBEV) oder das Japanische-Enzephalitis-Virus (JEV) gehören. Sie sind alle gekennzeichnet durch etwa 50 – 60 nm große, ikosaedrische und von einer Lipidmembran umhüllte Partikel mit einem Nukleokapsid von etwa 30 nm Durchmesser. Dieses enthält die einzelsträngige, positiv orientierte, ~11 kb lange RNA, deren *open reading frame* zu einem Polyprotein translatiert wird [6]. Enzymatisch wird es durch virale und zelluläre Proteasen in drei Struktur- (Capsid (C), Membran (prM/M)

und *Envelope* (E)) und 7 Nichtstrukturproteine (NS1, NS2A, NS2B, NS3, NS4A, NS4B, und NS5) gespalten. Die Strukturproteine E und M sind in der Lipidmembran der Viruspartikel verankert. In unreifen, nicht infektiösen Viren bilden sich 60 Trimere von E-prM Heterodimeren aus, welche sich zu einer stachelförmigen Oberfläche anordnen, bei der prM an den Spitzen exponiert ist (siehe Abb. 1). Durch die Exozytose passieren die unreifen Viruspartikel das trans-Golgi Netzwerk, in dem der leicht saure pH-Wert eine Konformationsänderung auslöst, welche eine Neuanordnung der E-Proteine und die Spaltung von prM zu pr und M durch eine zelluläre Furinprotease hervorruft [7]. Die so gereiften, infektiösen Viren bilden in der neutralen pH Umgebung der extrazellulären Matrix glatte Oberflächen aus [8]. Auf dieser sind die E-Proteine in 90 homodimeren fischgrätenartig parallel zur Membran angeordnet [9]. Diese fundamentalen strukturellen Eigenschaften sind zwischen allen Flaviviren konserviert [10].

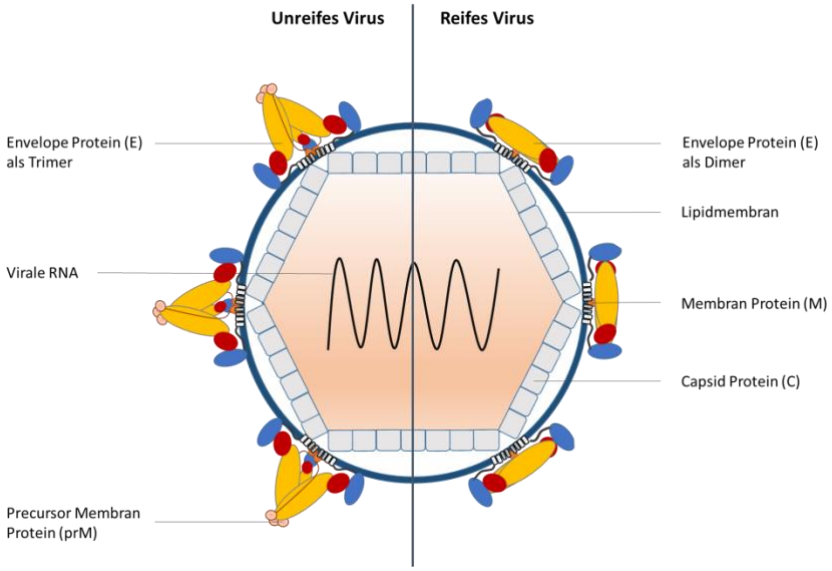

Abb. 1: Struktur eines Flavivirus. *Das Virus besteht aus einem ikosaedrischen Kapsid, welches die positv orientierte RNA umhüllt. Im unreifen Zustand sind in der Membran die strukturellen Proteine E und prM als Heterodimere verankert, welche sich zu Trimeren anordnen. Ist das Virus gereift, bilden sich E-Homodimere auf der Oberfläche aus. In Anlehnung an:* [11]

ZIKV ist Teil des Spondweni Serokomplexes [12], welcher durch die Kreuzneutralisation polyklonaler Seren bestimmt wird. Phylogenetisch wird ZIKV in eine afrikanische und eine asiatische Linie aufgeteilt. Die afrikanische Linie beinhaltet Stämme aus Zentral- und Westafrika, wohingegen der asiatischen Linie Isolate aus Asien und die Zika-Stämme aus Südamerika zugeordnet werden [13,14]. Wenn man die Aminosäuresequenz des NS5 Proteins vergleicht, ergibt sich ein Cluster von ZIKV und dem JEV Serokomplex [15]. Zieht man jedoch die Aminosäuresequenzen des E-Proteins für den Vergleich heran, ist ZIKV mit ca. 55 % Identität näher zum DENV-Serokomplex verwandt, welcher aus den

Hauptserotypen 1-4 besteht. Jeder der DENV-Serotypen kann nochmals durch Analyse der E-Protein Sequenz oder des gesamten Genoms in Genotypen aufgeteilt werden. Die größte Variabilität bildet dabei DENV-2 mit 6 Genotypen, gefolgt von DENV-1 und -3 mit jeweils 5 Genotypen und den 4 Genotypen des DENV-4 Serotyps [16].

1.3 Übertragung, Epidemiologie und Symptomatik

1.3.1 Dengue-Virus

In den tropischen und subtropischen Regionen der Welt gehören die vier DENV Serotypen zu den häufigsten arboviralen Infektionen des Menschen. Die Übertragung findet in einem direkten Zyklus durch einen Stich von Mücken der Gattung *Aedes* (hauptsächlich *Aedes aegypti* und *Aedes albopictus*) statt [17,18]. Alle vier DENV-Serotypen zirkulieren sowohl in einem urbanem als auch sylvatischen Zyklus, welche evolutionär und ökologisch voneinander getrennt sind [19]. Der sylvatische DENV-Zyklus findet in Regionen Südostasiens und Westafrikas statt, in dem nichthumane Primaten die einzige Ausbreitungs- und Reservoirquelle darstellen [20]. In seltenen Fällen kommt es in ländlichen Regionen zu einem Transfer zum humanen Zyklus [16]. Jedoch sind die zirkulierenden DENV-Stämme, die für die großen Epidemien unter Menschen verantwortlich sind, ausschließlich im humanen Zyklus vertreten. Die erste Annäherung an den Menschen fand vermutlich in Asien aufgrund von Waldrodungen im Zuge der Anpassung an die landwirtschaftliche Bodennutzung statt. Die Seefahrt und der Sklavenhandel während des 17. Jahrhunderts ermöglichten weiterhin die Ausbreitung der anthropophilen afrikanischen *A. aegypti* als Vektor nach Asien und in die Neue Welt [21]. Zu den ersten größeren Epidemien, bei denen auch zum ersten Mal das Dengue-hämorrhagische Fieber klinisch beschreiben wurde, kam es nach dem zweiten Weltkrieg auf den Philippinen und in Thailand [22,23]. In den 1970er Jahren wurde im Zuge der Beendigung des DDT-Einsatzes, die Wiederverbreitung der *Aedes* und die Einfuhr von Dengue in Südamerika verzeichnet [24]. Seit dem wird eine hyperendemische Verbreitung von allen vier DENV-Serotypen in Südostasien, Südamerika und Afrika beobachtet [25]. Die regionale Ausbreitung der Serotypen schwankt zyklisch über die Zeit, wobei, aufgrund der unzureichenden Kreuzneutralisierung, ein Serotyp den anderen über die Jahre abwechselt [26]. Die Symptomatik hängt von verschiedenen Faktoren, wie z. B. der Virulenz des infizierenden Stammes, dem Alter des Patienten oder von bereits bestehenden kreuzreaktiven Antikörpern aus einer vergangenen DENV-Infektion ab [27]. Laut Schätzungen verlaufen ca. 70 – 75 % der Infektionen inapparent oder sehr mild, so dass sie nicht gemeldet werden. Symptomatische Infektionen zeichnen sich im Allgemeinen durch das Dengue-Fieber (DF) aus, welches grippe-ähnlich mit einem plötzlichen Fieberschub, Arthralgie, Myalgie, Exanthemen und retro-orbitalen Kopfschmerzen auftritt. Ca. 1 – 2 % der Infektionen verlaufen in der schwersten Form des Dengue-hämorrhagischen Fiebers (DHF) oder des Dengue-Schock Syndroms (DSS). Diese

Krankheitsverläufe rufen jährlich ca. 20.000 letale Fälle hervor und äußern sich durch allgemeine Blutungsneigung, Ausbildung von Petechien, Blutdruckabfall, gastrointestinalen Blutungen, Zyanose und Hepatomegalie [28]. Hierfür stellen vor allem Kinder in Hyperendemiegebieten und sekundär infizierte eine Risikokruppe dar [29].

1.3.2 Zika-Virus

Wie bei DENV, erfolgt auch die direkte Übertragung von ZIKV auf Menschen durch den Stich einer Mücke des Genus *Aedes* als Vektor. Die Hauptspezies bei der Übertragung und Ausbreitung stellen, wie bei DENV, die anthropophilen tropisch und subtropisch beheimateten *A. aegypti* und *A. albopictus* dar [30–34]. Dabei ist die Übertragung durch die am weitesten verbreitete Spezies *A. albopictus,* welche auch in mediterranen Regionen Europas heimisch ist, weniger effizient [35]. Neben dem urbanen Übertragungsweg, existiert auch für ZIKV in afrikanischen und asiatischen Gebieten ein sylvatischer Zyklus, bei dem nichtmenschliche Primaten das Virusreservoir darstellen [20,36]. Weiterhin wurde im Zuge der Ausbreitung von ZIKV über den pazifischen Raum und in die westliche Hemisphäre auch die Übertragung durch Blut- oder Organspenden in der Transfusionsmedizin nachgewiesen [37,38]. Außerdem gibt es mehrere Berichte über die perinatale [39] und sexuelle Übertragung von ZIKV [40–44].

Die zuletzt genannten Übertragungswege nehmen jedoch aus epidemiologischer Sicht eine untergeordnete Rolle ein. Vielmehr ist die Ausbreitung von ZIKV, wie bei DENV, eng an die Verbreitungsgebiete der *Aedes* Mücken geknüpft, welche durch die zunehmende Urbanisierung und Globalisierung stetig zunehmen. ZIKV wurde erstmalig 1947 im Zika Wald in Uganda aus einem fiebernden Rhesus Affen isoliert und wenig später erfolgte der Nachweis in einem *A. africans* Moskito [30]. Die ersten menschlichen Infektionen wurden 1954 in Nigeria beschrieben [45]. Seither gab es sporadische Fälle in Afrika und Asien, wobei nur einzelne Infizierte milde Krankheitssymptome entwickelten, wie z. B. mildes Fieber, Kopfschmerzen und Maläse. Der erste bekannte große Ausbruch fand 2007 durch den asiatischen Genotyp auf der Insel Yap in Mikronesien statt, bei dem schätzungsweise 73 % der Einwohner infiziert wurden und 20 % davon Symptome entwickelten [46]. 2013 folgte ein Ausbruch in Französisch Polynesien, bei dem sich bis zu 32.000 Menschen infizierten, gefolgt von der Ausbreitung auf Ozeanien, Neukaledonien und den Cook Inseln [47]. In Brasilien wurden die ersten autochthonen ZIKV Infektionen Anfang 2015 beschrieben, bei denen der asiatische Genotyp identifiziert wurde, welcher eng mit den Isolaten aus Französisch Polynesien verwandt war [48,49]. Seit der Einführung des ZIKV im Nordosten Brasiliens breitete sich die Epidemie über das gesamte Land aus und weitete sich im Jahr 2016 auf viele Länder Süd- und Mittelamerikas aus [50]. Die Symptomatik bestand aus den vorher genannten milden grippe-ähnlichen Anzeichen, Arthralgie, Exanthemen, Konjunktivitis und weniger häufig auftretenden Beschwerden wie Ödemen, retroorbitalen Schmerzen und Lymphadenopathien. Zum ersten Mal

erfolgte auch die Assoziation einer ZIKV-Infektion mit kongenitalen Fehlbildungen von Neugeborenen, wie z. B. Mikrozephalie, Arthrogryposis, Netzhaut- und Sehnervanomalien und weiteren Defekten, welche als kongenitales Zika-Syndrom zusammengefasst werden [51–54]. Weiterhin werden das Guillain-Barré Syndrom und andere neurologische Erkrankungen wie Meningoenzephalitis oder Myelitis mit ZIKV-Infektionen verbunden, welche in seltenen Fällen auftreten [55,56].

1.4 Das flavivirale *Envelope* Protein

Das *Envelope* (E) Protein ist ein, aus drei Strukturdomänen bestehendes, Klasse-II virales Fusionsprotein, welches das Ziel der effektivsten neutralisierenden Antikörper der humoralen Immunantwort darstellt. Funktional ist es für die Rezeptorbindung und für die Membranfusion in der Wirtszelle verantwortlich, wodurch es Bestandteil vieler antiviraler Therapieansätze, Impfstoffstrategien und aufgrund der hohen Immunogenität auch Bestandteil diagnostischer Entwicklungen ist. In Flaviviren ist das E-Protein in der Aminosäuresequenz zu ca. 40 % identisch, weshalb die Faltung und der strukturelle Aufbau konserviert sind. Basierend auf Röntgenkristallographie der N-terminalen Ektodomäne, kann man das E-Protein in die β-Fass geformten strukturellen Domänen I-III (E-DI-DIII) unterteilen (siehe Abb. 2) [57,58]. E-DI ist eine diskontinuierliche Domäne und stellt das Verbindungsglied zwischen E-DII und E-DIII dar, mit denen sie durch flexible Gelenke verbunden ist. Hier befindet sich ein, in vielen Flaviviren konserviertes, N-glykosyliertes Asparagin an der Stelle 153 in Dengue und 154 in Zika-Viren. Dieses schirmt vermutlich die benachbarte konservierte E-DII Fusionsschleife ab und stabilisiert die Dimerbindung. Diese Glykosylierung wurde daher mit der Pathogenität und dem Zelltropismus in Verbindung gebracht [59,60]. So ist bei ZIVK das Glykan an dieser Stelle länger als bei DENV und hat größere Ähnlichkeit zu WNV, bei dem diese Modifikation mit Neurotropismus im Zusammenhang steht [61]. Einzigartig für DENV ist die Glykosylierung des Asparagins an Stelle 67 in der Domäne II, welche für die direkte Rezeptorbindung mit dem Mannose-spezifischen DC-SIGN Komplex der Rezeptorzelle verantwortlich ist [62–65]. Die Dimerisierungsdomäne E-DII ist durch Disulfidbrücken vernetzt, welche die cd-Schleife an der Spitze des Monomers stabilisieren. Sie enthält das unter Flaviviren stark konservierte Fusionspeptid DRGWGNGCGLFGGK [58]. Im dimerisierten E-Protein liegt die Fusionsschleife (*fusion loop* – FL) in einer hydrophoben Tasche, welche von hydrophilen Epitopen umgeben ist. Bei einem niedrigen pH Wert wird die hydrophobe Spitze von E-DII exponiert, welche dann mit der Wirtszellmembran verschmelzen kann und wodurch das Nukleokapsid in die Zelle entlassen wird [7,57,66]. Nahe dem C-Terminus befindet sich die, an der Virusoberfläche exponierte, Immunglobulin ähnlich gefaltete, kontinuierliche Domäne E-DIII, die durch die Sequenz RGE/RGD für die Bindung an zelluläre Integrin Rezeptoren verantwortlich ist [67]. Studien haben gezeigt, dass die effektivsten neutralisierenden Antikörper gegen Epitope

von E-DIII gerichtet sind [68–70]. Die letzten 20 % der C-terminalen E-Protein Sequenz bildet die *stem-anchor*-Region, welche das Protein durch amphiphile Alpha Helices mit der Membran verankert. Weiterhin interagiert sie mit dem prM Protein während der Virusreplikation und ist für die strukturellen Veränderungen bei der pH-induzierten Trimerbildung während des Fusionsvorgangs mit der Wirtsmembran beteiligt [71,72].

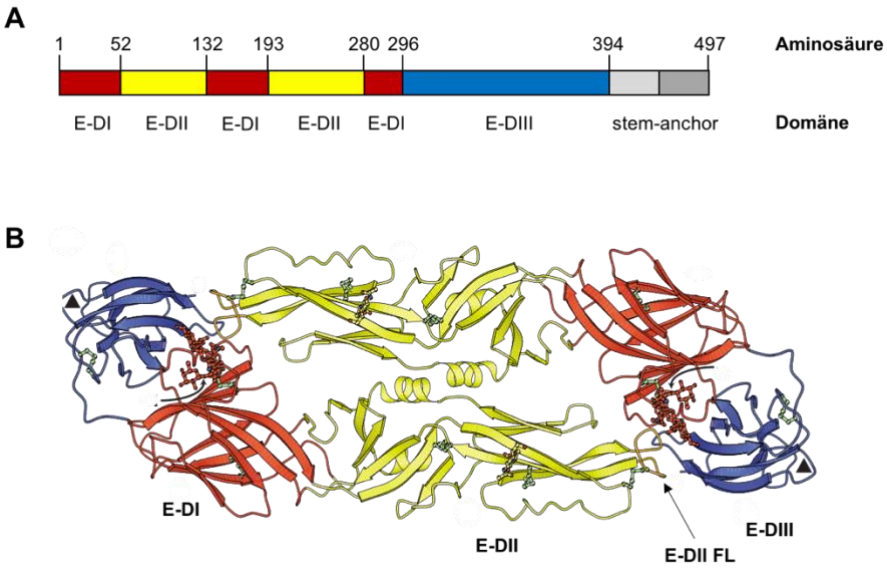

Abb. 2: Die Struktur des Envelope Proteins: (A) *Das flavivirale E-protein ist in einer Domänenstruktur organisiert, wobei sich die Domänen E-DI und E-DII diskontinuerlich auf der Aminosäuresequenz befinden und erst durch die tertiäre Struktur gebildet werden. Die Domänen E-DIII und stem-anchor liegen kontinuierlich im C-Terminus der Sequenz.* ***(B)*** *Die strukturellen Domänen werden im 3-D Modell sichtbar, welches das E-Protein als antiparalleles Homodimer zeigt, so wie es im reifen Viruspartikel vorliegt. Am distalen Ende von E-DII ist die Fusionsschleife (FL) lokalisiert. Modifiziert nach* [57].

1.5 Humorale Immunantwort

Die humorale Immunantwort einer DENV Infektion ist gekennzeichnet durch einen Großteil Serotyp- und Flavivirus-kreuzreaktiver Antikörper, die jedoch kein oder nur ein geringes neutralisierendes Potential besitzen. In der konvaleszenten Phase werden dann vermehrt funktionale, langlebige und Serotyp-spezifische, neutralisierende Antikörper gebildet, welche gegen den homologen Serotyp eine lebenslange Immunität vermitteln [73–75]. Studien haben belegt, dass durch die große Fraktion kreuzreaktiver Antikörper der primären Immunantwort in früh-konvaleszenten Seren, ca. sechs Monate alle DENV Serotypen und teilweise auch ZIKV durch Bildung von großen Immunkomplexen, kreuz-neutralisiert werden [76,77]. Dadurch entsteht für eine kurze Zeit ein umfassender Schutz gegen heterologe DENV-Serotypen [78]. Kommt es danach zu einer Sekundärinfektion mit einem heterologen DENV-Serotyp, wird das, in mehreren Studien postulierte, „*original antigenic sin*“ Phänomen beobachtet [79,80]. Hier ist die B-Zell

Antwort von der Aktivierung und Expansion kreuzreaktiver B-Gedächtniszellen dominiert, die zwar den infizierenden Serotyp erkennen, jedoch aus der Primärinfektion hervorgegangen sind [81]. Diese produzieren kreuzreaktive Antikörper, die mit hoher Affinität an den primären und sekundären Serotyp binden können, aber eine höhere Avidität zu dem Primär-Serotyp aufweisen. Weiterhin wird ein geringer Anteil Serotyp-spezifischer, neutralisierender Antikörper gebildet [82]. Im Allgemeinen zeichnet sich eine sekundäre DENV-Infektion durch einen schnelleren und steileren Anstieg des IgG-Spiegels im Vergleich zu Primärinfektionen aus [83]. Außerdem gibt es Unterschiede in den Isotypen gebildeter Antikörper. Bei der ersten DENV-Infektion können ab dem 5. Tag und bis zu 3 Monaten nach Symptombeginn IgM-Antikörper nachgewiesen werden, wogegen in Sekundärinfektionen die IgM-Antwort sehr heterogen verläuft, und nur geringe oder nicht messbare IgM-Titer verzeichnet werden [84,85].

Charakteristisch für DENV-Sekundärinfektionen ist vor allem die erhöhte Pathogenität, welche sich vermehrt durch DHF/DSS äußert [86,87]. Hervorgerufen wird dieses Phänomen durch die Antikörper vermittelte Infektionsverstärkung (*antibody dependent enhancement*—ADE), bei der bereits existierende Antikörper aus der Primärinfektion oder maternale Antikörper zwar an das heterologe Virus binden können, jedoch nicht ausreichend avide oder konzentriert sind, um eine neutralisierende Wirkung hervorzurufen [88]. Stattdessen werden die infektiösen Viren opsoniert und durch Fcγ-Rezeptor tragende Monozyten, Makrophagen oder Dendridischen Zellen (Wirtszellen) internalisiert, wodurch die Viruslast insgesamt zunimmt [89,90]. Die erhöhte Viruslast führt im Weiteren auch zu einer Zunahme in der Zytokinproduktion der infizierten Zellen, die einem „Zytokinsturm“ gleichen kann und die erschwerte Symptomatik wie erhöhte Blutungsneigung hervorruft. Durch die hohe Sequenz- und Oberflächenhomologie zwischen E-Proteinen von DENV und ZIKV und die gleichzeitig limitierte Kreuzneutralisation der hervorgerufenen Antikörperantworten gehen Studien davon aus, dass ADE auch bei ZIKV-Infektionen in DENV-Endemiegebieten möglich ist [91,92]. In diesen Regionen beträgt die DENV-Seroprävalenz bis zu 90 %, wodurch das neu aufgetretene ZIKV dort als sekundäre Flavivirusinfektion erscheinen kann. Sowohl *in-vitro*, als auch *in-vivo* wurde bereits belegt, dass DENV-Antikörper ZIKV-Infektionen und damit auch die Pathogenese verstärken können [76,93,94]. Die erschwerte Symptomatik inkl. Mikrozephalie und die rasche Ausbreitung von ZIKV in DENV-Endemiegebieten Südamerikas sind eventuell in diesem Zusammenhang zu sehen. Jedoch fehlen hierfür noch epidemiologische Studien, die diese Theorie vollständig in humanen Populationen nachweisen.

Analysen mit polyklonalen Seren und humanen monoklonalen Antikörpern von DENV- und ZIKV- infizierten Patienten haben ergeben, dass die Antikörperantwort mehrheitlich Epitope der Strukturellen Proteine prM und E, sowie NS1 erkennt. Ein großer Teil der DENV-Antikörperantwort ist gegen die hoch konservierte FL in der E-DII Domäne gerichtet [95,96]. Diese Antikörper sind stark kreuzreaktiv und haben ein sehr niedriges

neutralisierendes Potenzial [74,97]. Stattdessen wurde gezeigt, dass diese Art von Antikörpern ADE stimulieren kann [73]. Monoklonale Antikörper von ZIKV infizierten Patienten haben ergeben, dass insbesondere die E-DI/DII Antikörper zu über 60 % mit den entsprechenden DENV-Antigenen kreuzreagieren, wogegen isolierte E-DIII Antikörper zu einem Großteil spezifisch sind und das Virus neutralisieren können [93]. Weiterhin werden stark neutralisierende, spezifische Antikörper gegen Epitope auf der Virusoberfläche gebildet, die zwei E-Proteine umspannen und nicht an lösliche E-Proteine (Monomere) binden können [15,91,98,99]. Bei dieser Art von Antikörpern wurde eine Kreuzneutralisation zwischen ZIKV und DENV beobachtet, weswegen sie sich im Fokus von Therapeutika- und Impfstoff-Entwicklungen befinden [77]. Ein weiteres Ziel der Immunantwort ist das prM-Protein, welches bei unreifen oder partiell reifen Viruspartikeln auf der Oberfläche exponiert ist. Bei DENV-Infektionen sind ca. 30 % der Antikörper gegen Epitope auf dem prM-Protein gerichtet. Es wurde gezeigt, dass sich dieser Anteil bei sekundär infizierten DENV-Patienten weiter erhöht und dass davon der größte Teil ADE induzieren kann, jedoch das Virus nur bedingt neutralisiert [100]. Gegen NS1 gerichtete Antikörper sind mehrheitlich typspezifisch [74]. Sie erkennen sowohl an der Wirtszellmembran gebundenes als auch in der extrazellulären Matrix lösliches NS1, welches in sehr hohen Dosen in akuten DENV-Seren nachgewiesen werden kann. Die entstandenen Antigen-Antikörperkomplexe beschleunigen die Komplementaktivierung, welche jedoch die Pathogenese erschweren kann. Beispielsweise kann Endothelzellmembran-gebundenes NS1 durch die Komplementsystemaktivierung und die damit verbundene Anaphylatoxinproduktion diese aufreißen und Blutungen im DHF/DSS hervorrufen [101,102].

1.6 Labordiagnostik von DENV- und ZIKV-Infektionen

1.6.1 Direkte Diagnostik

Der direkte Nachweis einer DENV- oder ZIKV-Infektion erfolgt in erster Linie durch die Detektion der extrahierten viralen RNA aus dem akuten Serum, Urin oder Speichel durch verschiedene RT-PCR oder *nucleic acid sequence based amplification* (NASBA) basierte Systeme. Die Sensivität dieser Methoden ist stark von der Virämie im Serum abhängig (siehe Abb. 3 und Abb. 4), welche bis zu 8 Tagen nach Symptombeginn anhält, bei DENV-Infektionen aber nach dem 4. Tag rapide absinkt [103–105].

Deutlich länger kann die virale RNA in Urin-, Speichel- oder bei ZIKV auch in Samenproben detektiert werden, was jedoch Testsysteme mit sehr niedriger Nachweisgrenze erfordert [106–108]. Der größte Vorteil dieser Methoden ist die hohe Spezifität, bei der, in Abhängigkeit der gewählten Sequenz, ein Nachweis von einzelnen Stämmen für epidemiologische Studien möglich ist. In der kurzen virämischen Phase kann das Virus auch direkt aus Blut, Serum oder Plasma isoliert und in Zellkultur, wie z. B. in

Moskito- C3/C6 oder den Säugetierzellen Vero, propagiert werden. In der ZIKV-Diagnostik wurden infektiöse Viren außerdem in Speichel-, Samen-, Urin-, Muttermilch-, Cerebrospinal- und Amnionflüssigkeiten nachgewiesen [41,109–111]. Diese Methodik dient trotz der hohen Spezifität und niedrigen Nachweisgrenze meist nur zu Forschungszwecken und wird aufgrund der hohen Anforderungen an Personal, Sicherheit sowie Laborausstattung, nicht in der Routinediagnostik angewandt.

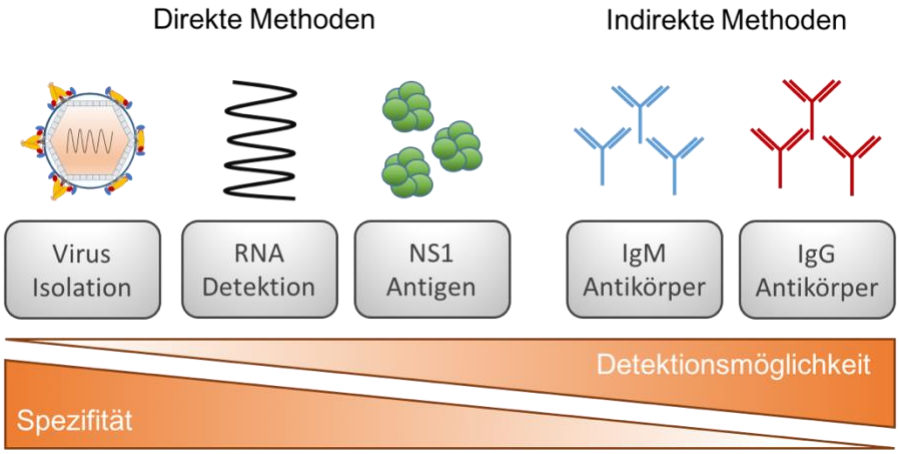

Abb. 3: Vergleich der direkten und indirekten Methoden in der DENV- und ZIKV-Diagnostik. *Die praktikabelsten Testmethoden haben die größte Detektionswahrscheinlichkeit, jedoch die geringste Spezifität. Der direkte Nachweis des NS1 Antigens erfolgt bisher nur in der DENV-Diagnostik. Adaptiert und modifiziert nach* [112]

DENV-Infektionen können außerdem durch den direkten Nachweis des NS1 Antigens, welches von infizierten Wirtszellen in hohen Konzentrationen sezerniert wird, diagnostiziert werden [113]. Diese Tests können in der akuten Phase bis zu 8 Tagen nach Symptombeginn durchgeführt werden, wobei die Sensitivität mit Zunahme des Testzeitpunktes, aufgrund der sinkenden NS1 Konzentration im Blut, stetig abnimmt [114]. Insbesondere bei sekundären DENV-Infektionen verringert sich die Sensitivität dieser Testmethoden aufgrund von bereits zirkulierenden, kreuzreaktiven Antikörpern, die das NS1 aus dem Blut durch Immunkomplexbildung klären [115]. Vorteilhaft ist jedoch die hohe Spezifität und schnelle Durchführbarkeit der NS1-Antigen Tests, die bereits als Point-of-care Anwendungen verfügbar sind [116].

1.6.2 Indirekte Serologische Diagnostik

Die serologische Diagnostik von DENV- und ZIKV-Infektionen gehört zu den einfachsten, zeitsparendsten und kostenschonendsten Methoden, welche weltweit auch eine routinemäßige Anwendung finden. Das größte Problem dieser Assays stellt jedoch die umfangreiche Kreuzreaktivität von Flavivirus induzierten Antikörpern dar. Der Grad der

Kreuzreaktivität ist u. a. abhängig vom verwendeten Antigen. Während ganze Viruspartikel oder das E-Protein bei hohen Sensitivitäten die höchsten Kreuzreaktivitäten aufweisen, sind die Nichtstrukturproteine NS1 und NS5 spezifischer, jedoch weniger sensitiv [117–119]. IgM- und IgG-Antikörper können mittels ELISA, indirektem Fluoreszenz-Antikörpertest oder Virus-Neutralisationstest nachgewiesen werden [112,120].

IgM-Antikörper können in akuten Primärinfektionen erstmalig 3 bis 5 Tage nach Symptombeginn bei ca. 50 % der DENV-Patienten nachgewiesen werden. Danach können IgM-Antikörper bis zu 3 Monaten nach Symptombeginn und mit Sensitivitäten von bis zu 90 % detektiert werden, was jedoch stark von dem verwendeten Antigen und Testsystem sowie dem Testzeitpunkt abhängig ist [121–124]. In flaviviralen Sekundärinfektionen sinkt die Sensitivität IgM-basierter Test deutlich, da die IgM-Titer meist viel geringer und in einem kürzeren Zeitfenster nachweisbar sind (siehe Abb. 4). Auch die IgM-Spezifität kann bei Sekundärinfektionen wegen des „*original antigenic sin*“ Phänomens verringert sein [125]. Aufgrund der langen Persistenz der IgM-Antikörper, kann in DENV-Endemiegebieten ein positiver IgM-Test alleine keine akute Infektion nachweisen, weshalb stets die Serokonversion in aufeinanderfolgenden Proben gezeigt werden muss. Diese beinhaltet den Wechsel von IgM- zu IgG-Antikörpern im Blut sowie den vierfachen IgG-Titeranstieg [28]. IgG-Antikörper werden in Primärinfektionen ab 10 Tagen nach Symptombeginn nachgewiesen [126]. In Sekundärinfektionen ist der Anstieg des IgG-Spiegels deutlich steiler und kann bereits ab dem 1. Tag nach Symptombeginn detektiert werden (siehe Abb. 4). Die Unterscheidung von primären oder sekundären DENV-Infektionen kann auch durch Messungen der Avidität erfolgen, welche bei Sekundärinfektionen höher ist. Weiterhin kann ein niedriges Verhältnis von IgM- zu IgG-Signalen ein Hinweis für eine DENV-Sekundärinfektion sein [127]. Die Spezifität IgG-basierter Methoden ist besonders bei Sekundärinfektionen deutlich geringer im Vergleich zu IgM-Antikörpern, weshalb der zuverlässige Nachweis einer DENV- oder ZIKV-Infektion in DENV-endemischen Regionen, die Durchführung eines Virus-Neutralisationstests als Bestätigung erfordert [125,128]. Dabei gilt aufgrund von Kreuzneutralisationen zwischen Antikörpern aus akuten DENV- und ZIKV-Infektionen nur ein vierfacher Unterschied beider Titer (DENV gegenüber ZIKV oder umgekehrt) als eindeutiges Testergebnis. Wie bei den IgG-Nachweisen kann als Alternative auch der Anstieg spezifischer Neutralisationstiter in aufeinanderfolgend entnommenen Serumproben verwendet werden [125]. Ein Virus-Neutralisationstest gilt in der serologischen Flavivirus Diagnostik als Gold-Standard, weil dieser die größte Spezifität aufweist [129]. Jedoch erfordert dessen Durchführung den Umgang mit infektiösen Viren, welche im Falle von DENV nur in BSL-3 Laboratorien gehandhabt werden dürfen. Daher ist der Neutralisationstest in Referenzlaboratorien an hohe Anforderungen an die Sicherheit und die Personalausbildung, sowie an lange Inkubationszeiten geknüpft. Weiterhin können Neutralisationstiter verschiedener Referenzlabore aufgrund z. B.

unterschiedlicher Virusstämme schwer miteinander verglichen werden, was ein hohes Maß an Standardisierung erfordert [130].

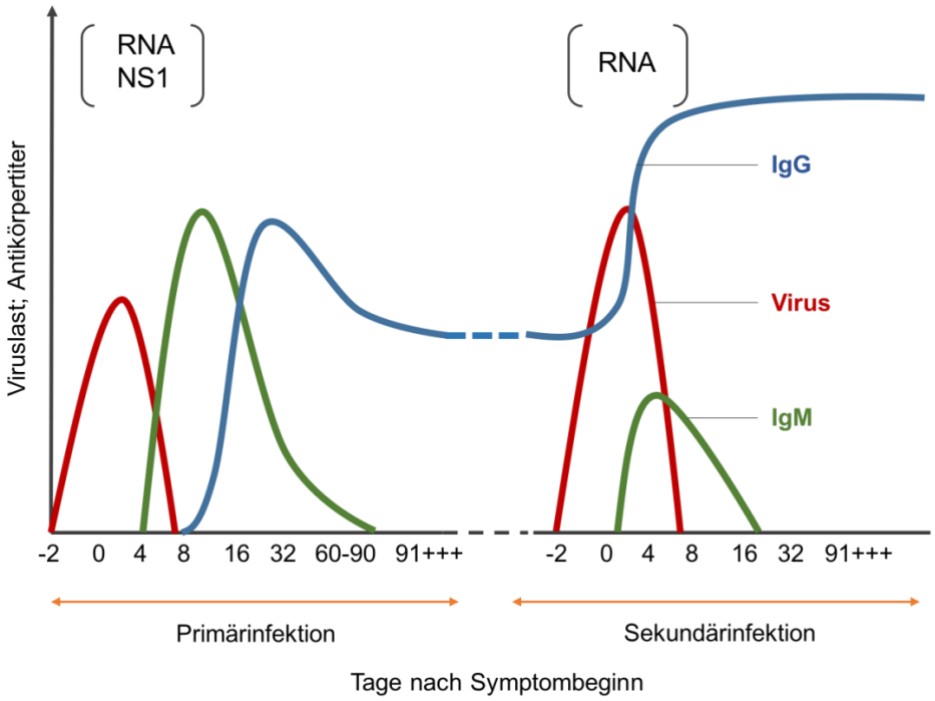

Abb. 4: Verlauf von diagnostisch relevanten Markern in flaviviralen Infektionen. *Der Titer von IgM- und IgG-Antikörpern variiert in Abhängigkeit von der Infektionsanzahl, wobei der IgM-Titer in einer Sekundärinfektion nur sehr gering ansteigt und IgG-Antikörper verstärkt werden. Die Virämie dauert in beiden Verläufen nur bis zu einer Woche und ermöglicht ausschließlich in diesem Zeitraum den Nachweis von viraler RNA und dem NS1 Antigen im Serum von DENV-Infektionen. Zuverlässig kann NS1 jedoch nur in DENV-Primärinfektionen detektiert werden. In Anlehnung an* [112]

1.7 Zielstellung

Die kreuzreaktiven Antikörper in der humoralen Immunantwort von DENV- und ZIKV-Infektionen sind vermehrt gegen die, in Flaviviren hoch konservierte, DII-FL Region des E-Proteins gerichtet. Besonders in DENV-Infektionen stellen diese Antikörper einen enormen Anteil dar. Eine Insertion von Mutationen in diesem Sequenzabschnitt führte bereits in Untersuchungen mit JEV und WNV *virus like particles* (VLPs) sowie in *E.coli* exprimierten WNV E-Proteinen zu einer verringerten Kreuzreaktivität mit heterologen Flaviviren [131–133].

Das erste Ziel der Arbeit war die serologische Charakterisierung von rekombinanten DENV E-Proteinen, welche durch vier Punktmutationen im Bereich der konservierten DII-FL Region modifiziert waren (DENV Equad). Hierfür sollten Equad Expressionskonstrukte aller vier DENV-Serotypen hergestellt werden, welche dann nativ in einem Insektenzell-basierten System hergestellt und aus dem Kulturüberstand aufgereinigt werden konnten. Die Strukturintegrität der rekombinanten Proteine sollte zunächst im Vergleich mit dem Wildtyp E-Protein (DENV Ewt) durch monoklonale Antikörper und im Weiteren mit polyklonalen Seren DENV-infizierter Patienten untersucht werden. Die Spezifität sollte mit negativen Referenzseren, sowie durch Seren aus heterologen flaviviralen Infektionen getestet werden. Hierfür sollte ein optimales Verhältnis aller DENV-Serotypen bestimmt werden, welches dann in einem IgM- oder IgG-basierten ELISA eine optimale Sensitivität und Spezifität für die DENV-Diagnostik erreichen kann.

Im Zuge der ZIKV-Epidemie in vielen Ländern Südamerikas, welche gleichzeitig als DENV-Endemie Gebiete gelten, war das zweite Ziel der Arbeit die Ausweitung der Thematik auf die serologische Diagnostik von ZIKV-Infektionen und vor allem die Unterscheidung zu Infektionen mit DENV. Hierfür sollte zunächst das ZIKV Equad Protein in *Drosophila* S2 Insektenzellen exprimiert, aus dem Kulturüberstand durch Affinitäts- und Größenausschlusschromatographie aufgereinigt und mit humanen ZIKV-, sowie heterologen flaviviralen Seren in IgM- und IgG-ELISAs untersucht werden. Weiterhin musste das bestehende DENV-Equad System durch Tests mit ZIKV positiven Seren neu evaluiert werden.

2 Publikationen

2.1 Publikation Nr. 1

Titel:
Recombinant Envelope-Proteins with Mutations in the Conserved Fusion Loop Allow Specific Serological Diagnosis of Dengue-Infections

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RESEARCH ARTICLE

Recombinant Envelope-Proteins with Mutations in the Conserved Fusion Loop Allow Specific Serological Diagnosis of Dengue-Infections

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Abstract

Dengue virus (DENV) is a mosquito-borne flavivirus and a major international public health concern in many tropical and sub-tropical areas worldwide. DENV is divided into four major serotypes, and infection with one serotype leads to immunity against the same, but not the other serotypes. The specific diagnosis of DENV-infections via antibody-detection is problematic due to the high degree of cross-reactivity displayed by antibodies against related flaviviruses, such as West Nile virus (WNV), Yellow Fever virus (YFV) or Tick-borne encephalitis virus (TBEV). Especially in areas where several flaviviruses co-circulate or in the context of vaccination e.g. against YFV or TBEV, this severely complicates diagnosis and surveillance. Most flavivirus cross-reactive antibodies are produced against the highly conserved fusion loop (FL) domain in the viral envelope (E) protein. We generated insect-cell derived recombinant E-proteins of the four DENV-serotypes which contain point mutations in the FL domain. By using specific mixtures of these mutant antigens, cross-reactivity against heterologous flaviviruses was strongly reduced, enabling sensitive and specific diagnosis of the DENV-infected serum samples in IgG and IgM-measurements. These results have indications for the development of serological DENV-tests with improved specificity.

Author Summary

The serological diagnosis of dengue is severely complicated by cross-reactivity between antibodies against different flaviviruses. Currently available tests cannot rule out false positive results due to infections or vaccinations with related pathogens such as West Nile virus or Yellow Fever virus. Most cross-reactive antibodies target the conserved fusion loop (FL) domain in the E protein (the major component of the viral envelope). Therefore, we generated insect-cell derived E proteins of DENV from the four different serotypes

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with mutations in the FL and set up an ELISA-based platform. Using these antigens we were able to detect DENV-infections with high sensitivity. In addition, cross-reactivity with a variety of heterologous flavivirus-infections was eliminated. The results have strong indications for the development of simple and sensitive serological DENV-tests with greatly improved specificity.

Introduction

Dengue virus (DENV) is a mosquito-transmitted pathogen of the family *Flaviviridae*, a group of small, enveloped and positive stranded RNA-viruses. Besides DENV there are several other human pathogenic vector-borne flaviviruses, such as yellow fever virus (YFV), West Nile virus (WNV), tick-borne encephalitis virus (TBEV) or Japanese encephalitis virus (JEV) [1]. DENV is endemic to over a hundred tropical and subtropical countries worldwide, and the numbers of annual infections are strongly increasing with a current estimate of 400 million [2]. High fever is the most common clinical symptom of a DENV-infection, but also severe complications are observed, such as dengue shock syndrome (DHS) or dengue haemorrhagic fever (DHF). Approx. half a million hospitalizations and several thousands of fatalities are caused by DENV every year [3,4]. Dengue viruses include four major distinct serotypes, named DENV-1 to DENV-4, and survival of an infection with one of these serotypes leads to a lifelong immunity to this serotype, but not to the others [5]. In parallel to the increasing distribution of its vector, mosquitos from the genus *Aedes*, DENV is emerging or re-emerging in several areas including Europe and North America. In Europe local transmission of the virus has been demonstrated in France and Croatia in 2010 [6,7]. In addition, a DENV-outbreak occurred in Madeira in 2012 and resulted in over 2000 cases and transportation of the virus into several other European countries [8].During the acute phase of infection, Dengue can be diagnosed by directly detecting viral RNA or the non-structural protein 1 (NS1), which is secreted by infected cells. About five days after onset of symptoms, these direct infection markers start to decrease in blood and DENV-IgM antibodies appear, followed by IgG a couple of days later. Therefore, except during the acute phase, DENV-infections are usually diagnosed by antibody-measurements, and several serological test systems are available [reviewed in 9,10]. Whereas IgM-detection can indicate recent primary infections, IgG-measurements are useful for detection of secondary infections, where IgM responses usually remain lower, and for serological surveillance activities. Acute dengue infections can be confirmed via a rise in IgM or IgG levels in paired samples [10]. One of the major problems in antibody-based diagnosis of dengue is the similarity of structural proteins of different flaviviruses which leads to cross-reactive antibodies and false positive test results [11–13]. Especially in areas where several flaviviruses co-circulate or in the context of vaccination against e.g. YFV or TBEV, this is of concern [1,14]. As the currently available tests cannot exclude flavivirus cross-reactivity, positive test results have to be confirmed by virus neutralization tests, which are time consuming and require BSL-3 laboratories. The E (envelope) protein is a major target of the human antibody response during DENV infections and is used in most available tests [15]. Cross-reactive antibodies target mainly the highly conserved fusion loop (FL) domain of the E protein which is involved in fusion of the viral and cellular membranes [16–19]. As a consequence, the insertion of mutations into the FL leads to a decrease in binding of cross-reactive antibodies, which has been employed to develop diagnostic methods on the basis of WNV- or JEV- virus-like particles (VLPs) [20–22]. Alternatively, other recombinant antigens than the E protein, such as NS1, have been used to differentiate flavivirus antibodies via titer-determinations [23].

It was shown previously that a bacterially expressed E protein from WNV bearing four point mutations in the FL and an adjacent loop domain can be used to serologically distinguish WNV- from TBEV and DENV-infections [22,24] by reducing the binding of antibodies from heterologous flavivirus infections. Here, we expand this technology to DENV. Quadruple mutant forms of the E proteins from the different DENV-serotypes, containing the same mutations as the WNV-protein [24], were generated in insect cells and analyzed as a diagnostic tool. The results show the high potential of this method for the development of a specific serological DENV-assay.

Materials and Methods

Cell culture

Drosophila S2 cells (Invitrogen) were propagated at 28°C in T-75 cm^2 flasks in Schneider´s medium supplemented with 10% FCS and 1% Pen/Strep (complete Schneider´s Medium).

Expression and purification of DENV envelope proteins

The sequences of the DENV-2 E wild type ectodomain (E-protein amino acid residues 1–399; strain 16681) and the quadruple mutants (Equad: T76R, Q77E, W101R; L107R) of DENV serotypes 1–4 (DENV-1: Nauru/West Pac/1974, E-protein amino acid residues 1–399; DENV-3: Sri Lanka/1266/2000, E-protein amino acid residues 1–397; DENV-4: Dominica/814669/1981, E-protein amino acid residues 1–399;) were synthesized (Centic Biotec) and cloned with BglII and EcoRI into the pMT/BiP/V5-His vector (Invitrogen). Plasmids were transfected with a Ca-Phosphate transfection kit (Invitrogen) according to manufacturer´s instructions into *Drosophila S2* cells. To generate stable cell lines 1 μg of pCoHygro (Invitrogen), containing a hygromycin resistance gene, was co-transfected with each expression vector. Stably transfected polyclonal S2 cell populations were generated after 3 weeks of selection with hygromycin B (300 μg/ml) in complete Schneider´s Medium. These cells were then propagated at 28°C in tissue culture flasks with complete Schneider´s medium containing 300 μg/ml hygromycin B and adapted to Sf900II medium containing 600 μg/ml hygromycin B. For an expression culture, cells were seeded at a cell density of 2–3 $x10^6$ cells/ml in 600 ml Sf900II medium in 2 l baffled Erlenmeyer shaker flasks at 28°C and 90 rpm and were induced with 700 μM CuSo4 at a cell density of 6 x 10^6 cells/ml. After 7 days the suspension culture was centrifuged for 15 min and 4000 g at 4°C and culture supernatant was concentrated and diafiltrated against His-binding buffer (20 mM sodium phosphate, 500 mM NaCl, 10 mM Imidazole, pH7,4) using Vivaflow 50R TFF cassettes (Sartorius) according to manufacturers´ instructions. The DENV E proteins were purified by immobilized metal affinity chromatography (IMAC) with 5 ml HisTrap FF crude columns (GeHealthcare) and size exclusion chromatography with a 16/600 HiLoad Superdex 200 pg column (GeHealthcare) using the ÄKTA pure 25 l chromatography system (GeHealthcare). Purified proteins were quantified using a BCA protein assay (Pierce). Conformation of the E-proteins and loss of the FL-domain structure in the Equad mutants were verified by binding studies of monoclonal antibodies recognizing DENV-2 epitopes in the domains DI-DII (antibody DV2-44), DIII (DV2-76, DV2-96, DV2-106) and FL (DV2-29) and WNV-specific FL-antibodies (E18 and E60) [25,26] (S1 Table).

Serum samples

22 DENV-positive serum samples were obtained from Padova University Hospital (Italy). The confirmed DENV cases were international travelers returning from endemic countries with diagnosis of recent infection. 15 DENV-positive serum samples and one negative control were

obtained from Seracare Life Sciences (USA), and 8 DENV-infected serum samples and two negative controls were obtained from ZeptoMetrix Corporation (USA). Three DENV-infected and one WNV-infected samples were from the Robert Koch Institute (Berlin) as part of an external quality assessment study [12]. Serum samples positive for DENV antibodies all fulfilled one of the following criteria: (a) positive in DENV-specific RT-PCR, (b) positive for both DENV IgM and IgG, (c) positive in a DENV-specific virus neutralization test (VNT). Serum samples from confirmed WNV-infections as well as sera from TBEV-infected individuals and negative controls were derived from Italy (University of Padova). The WNV-positive samples (IgG and IgM) were from seroprevalence studies, blood donors or patients with WNV- neuroinvasive disease. WNV-infections were confirmed by VNT. TBEV IgG-positive serum samples were selected from a seroprevalence study in forest rangers with a history of confirmed TBEV infection and no previous exposure to DENV. Serum samples from YFV-vaccinated individuals (IgG positive) participating in a randomized controlled vaccination study were obtained from the Robert Koch Institute (Berlin, Germany). These were confirmed by VNT.

Ethics statement

Ethical approval for all serum samples from Padova University was obtained from the Padova University Hospital ethics committee. The randomized controlled vaccination study for YFV from the Robert Koch Institute was approved by the Charité medical ethics committee. All persons participating in this research provided informed consent and all samples were analyzed anonymously.

Antibody measurements

Indicated protein amounts of DENV-2 Ewt and Equad or DENV1-4 Equad mixtures were coated overnight on Nunc polysorb plates (Thermo Scientific) in 100 µl coating buffer (15 mM Na2CO3,35 mMNaHCO3 pH 9.6) at 4°C. The plates were washed three times with 350 µl per well of PBS-0,05% Tween and blocked with 200 µl of 5% non-fat milk powder (blocking solution) for 2 h at room temperature. After a second washing step, human sera were diluted 1:100 in 100 µl blocking solution per well and incubated for 1.5 h at room temperature. Following a third washing step, the HRP-conjugated secondary goat anti human IgG (Fisher Scientific, 1:10000 in 100 µl blocking solution per well) or rabbit anti human µ-chain IgM (Dianova, 1:7500 in 100 µl blocking solution per well) antibody was added for 1 h at room temperature. After a fourth washing step, 100 µl TMB substrate (Biozol) per well were incubated for 30 min at room temperature. The reaction was stopped with 50 µl 1 M H_2SO_4 and signals were read out at 450 nm with background reduction at 520 nm in a micro plate reader (Infinite M200, Tecan). All tests were performed in duplicates and in two independent experiments. The Panbio Dengue IgG Indirect ELISA and was used according to the manufacturer´s instructions.

Statistical analysis

All antibody measurements were performed in duplicates in at least two independent experiments. Graphical and statistical analysis of the data in boxplots was carried out using SigmaPlot.

Results

To enhance specificity of serological DENV diagnosis we inserted 4 amino acid point mutations into the conserved fusion loop (FL) domain and an adjacent loop domain of DENV wild-type (wt) E proteins, yielding quadruple mutants for DENV serotypes 1 to 4 (DENV 1–4

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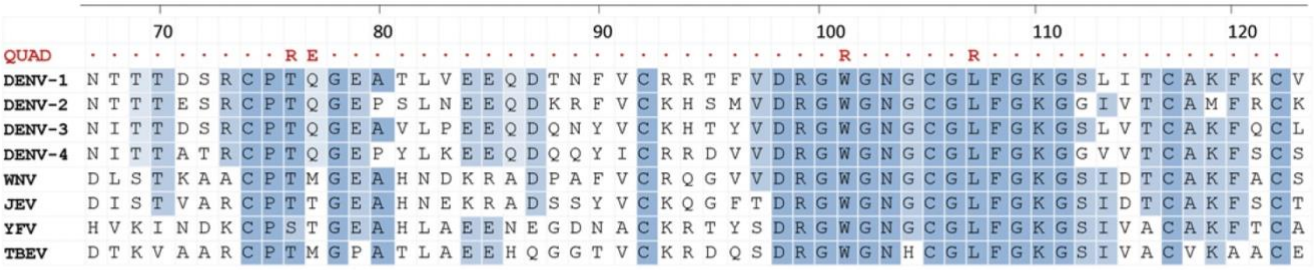

Fig 1. Alignment of the amino acid sequences containing the fusion loop domain from E proteins of DENV 1–4, WNV, JEV, YFV, TBEV and the four point mutations of the QUAD proteins. Amino acids numbering: 1 is start of the E protein.

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Equad-proteins, Fig 1). The mutant proteins as well as the DENV-2 wt E-protein were overexpressed in Drosophila S2 cells and secreted into the culture´s supernatant. After purification by immobilized metal affinity chromatography several unspecific proteins were co-purified. Therefore a second purification step using size exclusion chromatography was performed to eliminate the visible unspecific bands (Fig 2A).

To determine the optimal antigen concentration per well for an IgG-ELISA increasing amounts of the proteins DENV-2 Ewt and DENV 1–4 Equad were incubated with three sera of DENV- and WNV- infected or flavivirus-uninfected individuals, respectively (Fig 3). Signal saturation of DENV-positive sera was observed with an antigen amount of approx. 200 ng per well for DENV -1, 3 and -4 Equad and 300 ng per well for DENV-2 Ewt and Equad proteins.

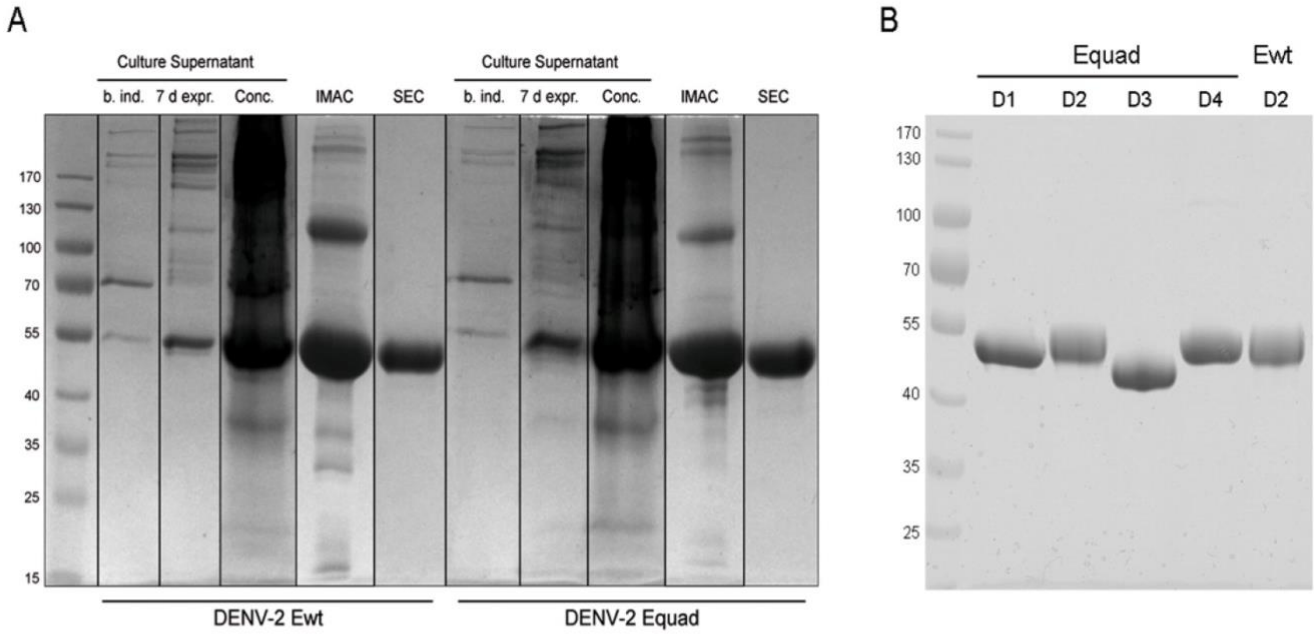

Fig 2. A: Expression and purification of DENV-2 Ewt and Equad from Drosophila S2 culture supernatants; supernatant before induction (b.ind.), after 7 days of expression culture (7 d expr.), concentrated via tangential flow (Conc.) and the two step purification with immobilized imidazole affinity (IMAC) and size exclusion chromatography (SEC) were separated on a 10% SDS-PAGE gel under reducing conditions. B: 6 μg of purified DENV1-4 (D1-D4) Equad and DENV-2 Ewt proteins were analyzed with SDS-PAGE. Proteins were stained with Coomassie blue. Size of molecular weight markers in kilo Daltons is indicated on the left.

doi:10.1371/journal.pntd.0004218.g002

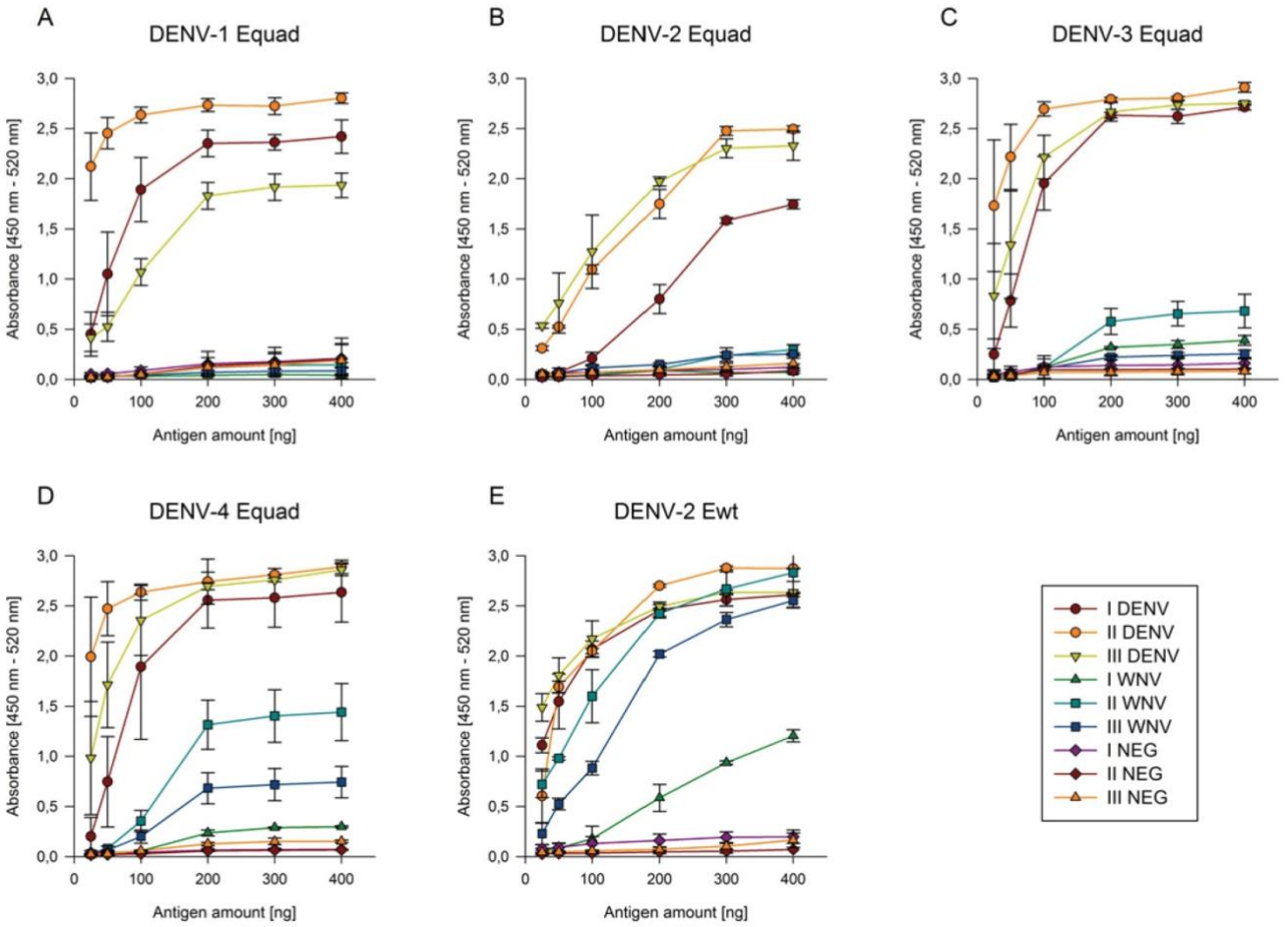

Fig 3. Antigen titration using the indicated amounts of DENV-1 (A), -2 (B), -3 (C) and -4 (D) Equad and DENV-2 Ewt (E) proteins with three different DENV, WNV and negative (NEG) human sera. Measurements were performed in duplicates in at least two independent experiments.

doi:10.1371/journal.pntd.0004218.g003

Two of the three DENV-positive sera showed a substantial decrease in binding to the DENV-1 and DENV-2 mutant proteins (Fig 3A and 3B) as compared to the DENV-2 wild type version, but the values were still detectable when 50 ng or more was used. Two WNV-positive samples bound to the wild type antigen as strongly as the DENV-positive sera, and the third started to show such cross-reactivity with more than 200 ng of antigen per well (Fig 3E). However, all WNV sera lost binding almost completely when the mutant antigens were used except for DENV-4 Equad, where two WNV-positive sera still showed substantial signals for 200 ng of antigen per well or more (Fig 3D). Negative sera showed negligible signal intensities for all proteins in all amounts tested. Based on these results an antigen amount of 300 ng per well was chosen for the analysis of a larger serum panel with DENV-2 wt and DENV-2 Equad in an IgG ELISA.

First, sera from 38 DENV-, 13 WNV-, 19 TBEV- infected, 8 YFV vaccinated and 7 uninfected individuals were incubated with DENV-2 Ewt protein (Fig 4A). Strong binding for DENV-positive samples was observed (mean absorbance value 2.36), however, several WNV- and TBEV- positive sera (mean values 1.26 and 0.82, respectively) showed cross-reactive

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signals which were in the range of DENV-infected sera. Samples from YFV-vaccinated individuals showed a reduced cross-reactivity in comparison to WNV- and TBEV-infected serum samples. When using the DENV-2 Equad protein, the cross-reactivities of WNV- and TBEV-infected samples were significantly reduced (mean values 0.106 and 0.160, respectively) (Fig 4B). In addition, the signal intensities of the DENV-positive samples changed. The mean value was reduced to 1.9 and the overall signal range increased from 2.1 to 2.4 in comparison to DENV-2 Ewt. Next, a mixture of the Equad proteins of all four serotypes was prepared (DENV 1–4 Equad Mix). Based on the titration curves (Fig 3) a total amount of 160 ng was found to be optimal in specificity and sensitivity, consisting of 50 ng of DENV 1–3 Equad respectively and 10 ng of DENV-4 Equad. The proportional amount of DENV-4 Equad was reduced compared to the other serotypes because it elicited a higher cross-reactivity with heterologous flaviviral sera (Fig 3D). The mixture was tested with the same serum panel. This resulted in an increase of the 25th percentile from 1.36 to 1.6, demonstrating a higher sensitivity compared to using only DENV-2 Equad. At the same time, cross-reactivity of WNV- and TBEV-positive sera was even further reduced (mean values 0.063 and 0.084, respectively) showing a higher specificity of the test in comparison to using DENV-2 Equad only. The stasticial analysis of the data is shown in S2 Table. The five DENV-positive sera detected as outliers on the lower end of the DENV-panel using the mutant antigens in Fig 4 were numbered. Whereas sera 1, 2 (both unknown DENV serotype infections) and 3 (DENV-3 infection) showed decreased binding to the DENV-2 Equad-protein as compared to the wild type, their signals increased when using the DENV 1–4 Equad mix. Also the signals of samples 4 and 5 (DENV-1 and -2 infections, respectively) decreased with the DENV-2 Equad compared to the wild type but were even lower with the mutant mixture (Fig 4C).

To compare these results with a state-of-the-art diagnostic method, a number of the serum samples were analyzed with a commercially available DENV-IgG ELISA. The test detected 3/3 DENV-positive sera as positive and 3/3 negative sera as negative. However, 10/10 WNV-infected sera were detected as positive with several OD-values similar to DENV-infected sera.

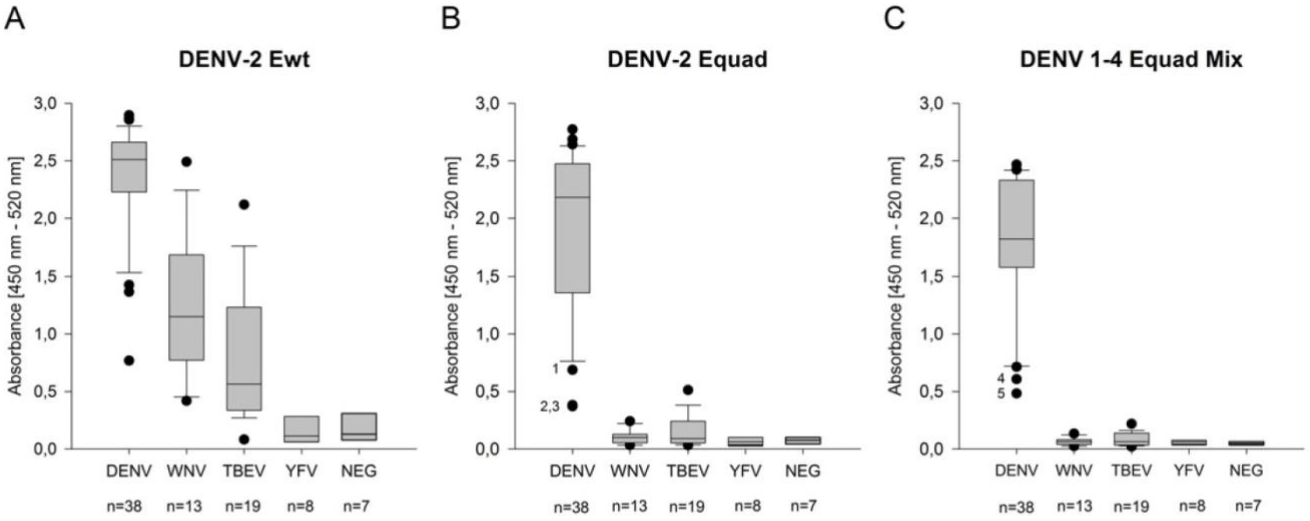

Fig 4. 300 ng per well of DENV-2 Ewt (A) and Equad (B) and 160 ng per well of a DENV 1–4 Equad mixture (C) were tested with DENV- WNV- and TBEV- infected and YFV-vaccinated sera compared to negative (NEG) samples in an IgG-ELISA (n = number of individuals). Bottom and top of the boxes are the first and third quartiles. The median signal is depicted as a line inside the box. Whiskers represent the 9th and the 91st percentile. Outliers in B and C are marked with numbers (1–5). Measurements were performed in duplicates in at least two independent experiments.

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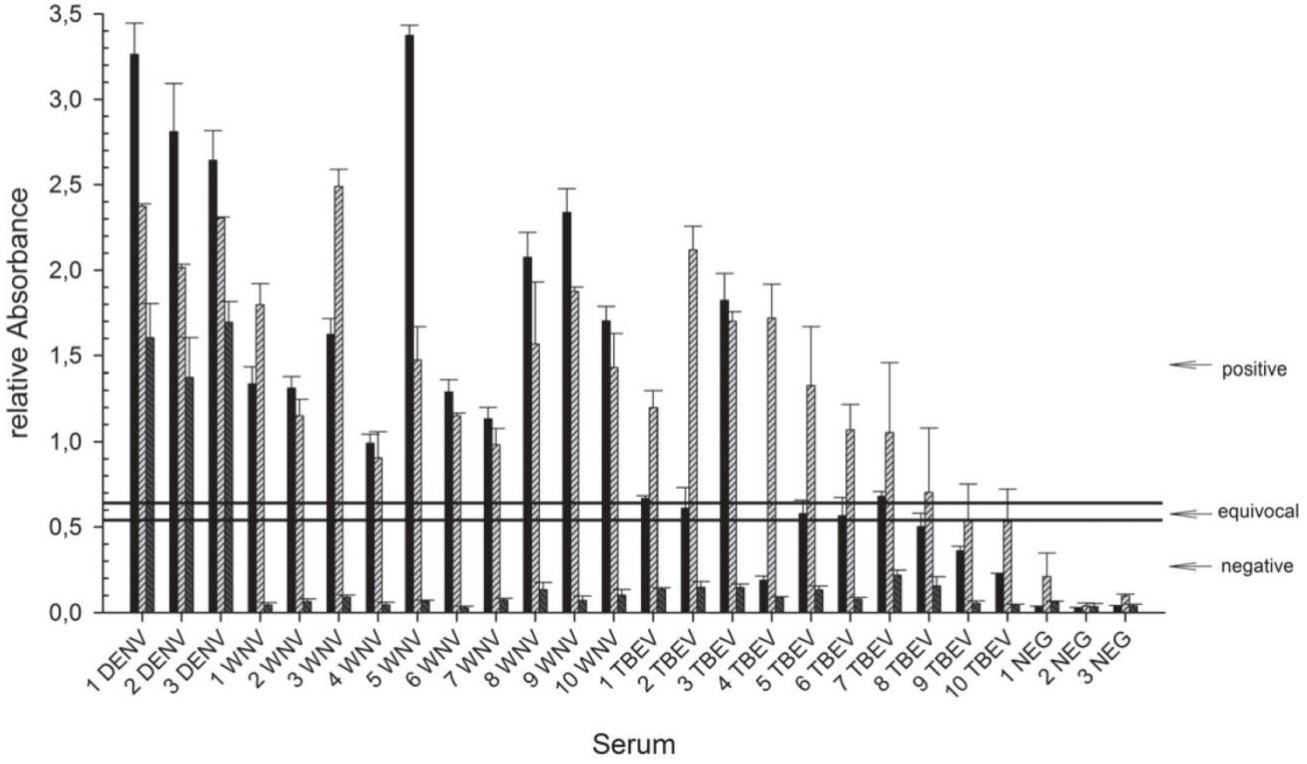

Fig 5. Comparison of different antigens for the detection of DENV IgG. Sera positive for IgG against DENV, WNV, TBEV or negative control sera were analyzed with the Panbio Indirect IgG ELISA (black), the DENV-2 Ewt protein (light gray, lined) or the DENV1-4 Equad mix (dark grey, lined). The absolute absorbance is indicated. Cut-Off values for the Panbio test were obtained by calculation of the internal standard of the manufacturer; these are indicated at the right and only refer to this test (horizontal bars: DENV-positive results with an OD-value higher than 1.1*cut-off, equivocal results having an OD-value between 1.1*cut-off and 0.9*cut-off, negative results with an OD-value lower than 0.9*cut-off).

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For TBEV-infected sera, 4/10 samples were detected as negative, 3/10 as equivocal and 3/10 as positive (Fig 5). Comparison with the values obtained using DENV Ewt showed a similar cross-reactivity. In contrast, when using the DENV 1–4 Equad mixture high signals were obtained only with the three DENV-infected sera (mean values of 1.5, 1.6 and 1.7, respectively) and cross-reactivity was strongly reduced in all samples from WNV- and TBEV-infections (mean values all >0,3, Fig 5).

Subsequently, the proteins were used to measure IgM antibodies in human sera. 300 ng of DENV 1–4 Equad mix were found to be optimal for IgM detection (S3 Table), which was then compared to 300 ng of DENV-2 Ewt in binding of IgM-positive DENV and WNV sera. Generally, the cross-reactivity of heterologous flavivirus IgM antibodies was lower than for IgG, as demonstrated by less binding to the Ewt protein (Fig 6A). By using the DENV 1–4 Equad mixture the mean value of signals for DENV sera was enhanced as compared for DENV-2 Ewt. On the other hand, WNV cross-reactivity was significantly reduced in comparison to DENV-2 Ewt (Fig 6B and S2 Table).

Discussion

A challenge for current serological dengue diagnosis is the high degree of cross-reactivity between antibodies produced during infections with related flaviviruses [27–29], leading to

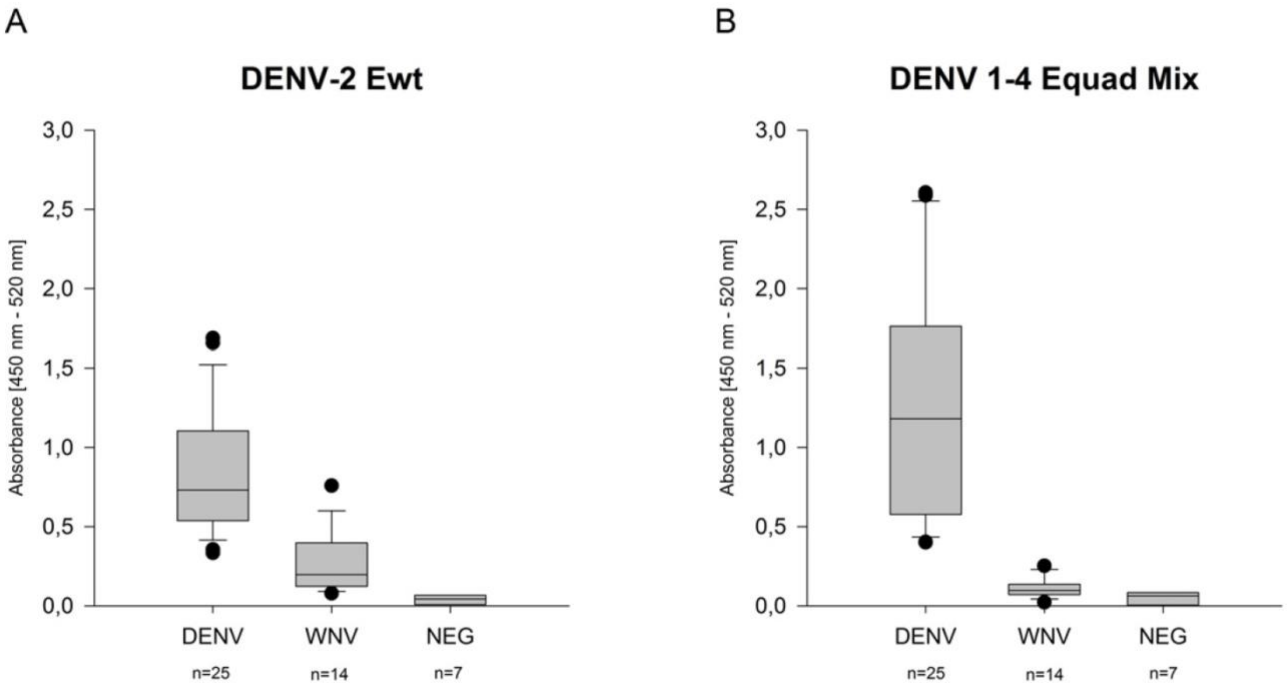

Fig 6. IgM-ELISA on 300 ng of DENV-2 Ewt (A) and DENV 1–4 Equad mixture (B) with different DENV, WNV and negative (NEG) sera (n = number of individuals). Bottom and top of the boxes are the first and third quartiles. The median signal is depicted as a line inside the box. Whiskers represent the 9th and the 91st percentile. Measurements were performed in duplicates in at least two independent experiments.

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false positive results in currently available serological test systems [11,12]. Especially in areas with different co-circulating flaviviruses, this is of concern [1,14]. Here, we present insect-cell derived recombinant DENV E proteins bearing mutations in the conserved FL domain to enhance the specificity of serological DENV diagnosis. When using the wild type E protein from serotype 2 (which is the basis for several available test systems) we found all the DENV-positive sera displayed high signals (Fig 4A). However, the cross-reactivity problem was also confirmed, as the signals obtained with many WNV- and TBEV positive samples were in the range of DENV-signals, although the mean intensities were lower (Figs 3E and 4A). As a consequence, several sera would be misdiagnosed as false-positive, which was indeed observed when a commercial assay was used (Fig 5).

It has been shown before that a large proportion of antibodies to DENV-infections are produced against the FL domain [17,18,22]. Accordingly, two out of three dengue sera showed a clearly decreased signal when the FL-mutant version of the E-protein from DENV-2 was used (Fig 3B and 3E). However, in the larger serum panel, the signals obtained with the DENV-sera remained detectable, but most of the binding of the heterologous flavivirus-antibodies was eliminated (Figs 3B and 4B). This result confirms previous studies performed with WNV and JEV, using VLP-based systems with single- or double mutations in the FL domain [20,21] and underlines the role of the conserved FL domain in cross-reactivity between flavivirus-antibodies. It also demonstrates that the four mutations of the Equad proteins from WNV [24] can be used to eliminate cross-reactive epitopes in DENV E proteins. When the mixture of all four serotype-specific mutant proteins was used, the values of many DENV-positive serum samples increased as compared to using DENV-2 Equad only, although only half the amount of total

protein was used (Fig 4C). This increase in sensitivity might result from serum samples that were from non-serotype 2 infections and now bound to their corresponding E-protein, such as the three outliers in Fig 4B. However, in the absence of knowledge of the infecting serotype, this interpretation remains speculative. Data on the infecting serotypes were available only for a limited subset of the DENV-positive sera in this study. In addition to an increase in sensitivity, the cross-reactivity of TBEV and WNV sera was further reduced with the serotype mixture and lower amount of antigen. Under the conditions used, all DENV-infected sera of the panel led to high absorbance values with the DENV 1–4 mix. Nevertheless, to avoid single outliers, the exact relative contribution and amount of each serotype mutant protein in the mixture might still be optimized. The titration curves (Fig 3) suggest that a doubling of the amount of the individual mutant antigens does still not lead to an increase in cross-reactivity.

Using the DENV 1–4 mix for IgM detection resulted in a higher mean value of positive samples as compared to the wildtype antigen (Fig 6). At the same time, the cross-reactivity with WNV-positive sera was decreased. Due to the generally observed higher specificity of IgM-antibodies [12], this improvement in specificity was less pronounced as for IgG-detection (Fig 6). Nevertheless, our data support the idea that recombinant E proteins bearing four point mutations are suitable antigens for the specific serological diagnosis of dengue. This is also supported by testing samples from a recent external quality assessment on serological dengue diagnosis [12], where the vast majority of participating laboratories (using various ELISA systems) detected a cross-reactive WNV serum as false positive. The serotype mixture used here was able to detect the samples with 100% accuracy (results are included in Fig 4), including diluted samples for sensitivity determination. In addition, the direct comparison of the DENV-1-4 mixture antigen with a commercially available test clearly showed the increased specificity of the mutant antigens (Fig 5). Although no cut-offs were yet defined for the DENV1-4 Equad, it is clear from Figs 4 and 5 that cross-reactive sera cause absorbance values more similar to flavivirus-negative than to DENV-positive samples.

We recently have performed a study using a bacterially expressed Equad mutant for WNV [24]. These data also showed a reduction in cross-reactivity. However, especially some DENV-positive samples with a high antibody titer still bound the mutant protein to some extent. Therefore, the ratio between signals obtained with the wildtype and mutant protein was calculated for each serum enabling correct diagnosis. In contrast, in the present study the mutant proteins alone were sufficient to exclude cross-reactivity, which might reflect higher sensitivity of the insect-cell derived antigen or specific differences between the antibody responses to WNV and DENV.

In summary, a system for the sensitive and specific serological diagnosis of DENV-infections is presented, which consists of recombinant E-proteins from the four major serotypes, with mutations in the FL and an adjacent loop domain. The binding of cross-reactive antibodies from heterologous flavivirus-infections is strongly decreased. These antigens might form a valuable basis for improved antibody tests, especially in areas where co-circulation of different flaviviruses is observed.

Supporting Information

S1 Table. Analysis of the recombinant DENV E proteins with monoclonal antibodies (mAb) recognizing conformational epitopes.
(DOCX)

S2 Table. Statistical analysis of data presented in Figs 4 and 6.
(DOCX)

S3 Table. Test of different antigen-amounts for measurement of IgM antibodies.
(DOCX)

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Author Contributions

Conceived and designed the experiments: AR SU. Performed the experiments: AR MP. Analyzed the data: AR SU LB MN GP. Contributed reagents/materials/analysis tools: LB MN GP. Wrote the paper: SU AR.

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2.2 Publikation Nr. 2

Titel:
Specific detection of dengue and Zika virus antibodies using envelope proteins with mutations in the conserved fusion loop

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ORIGINAL ARTICLE

Specific detection of dengue and Zika virus antibodies using envelope proteins with mutations in the conserved fusion loop

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Detection of antibodies is widely used for the diagnosis of infections with arthropod-borne flaviviruses including dengue (DENV) and Zika virus (ZIKV). Due to the emergence of ZIKV in areas endemic for DENV, massive co-circulation is observed and methods to specifically diagnose these infections and differentiate them from each other are mandatory. However, serological assays for flaviviruses in general, and for DENV and ZIKV in particular, are compromised by the high degree of similarities in their proteins which can lead to cross-reacting antibodies and false-positive test results. Cross-reacting flavivirus antibodies mainly target the highly conserved fusion loop (FL) domain in the viral envelope (E-) protein, and we and others have shown previously that recombinant E-proteins bearing FL-mutations strongly reduce cross-reactivity. Here we investigate whether such mutant E-proteins can be used to specifically detect antibodies against DENV and ZIKV in an ELISA-format. IgM antibodies against DENV and ZIKV virus were detected with 100% and 94.2% specificity and 90.7% and 87.5% sensitivity, respectively. For IgG the mutant E-proteins showed cross-reactivity, which was overcome by pre-incubation of the sera with the heterologous antigen. This resulted in specificities of 97.1% and 97.9% and in sensitivities of 100% and 100% for the DENV and ZIKV antigens, respectively. Our results suggest that E-proteins bearing mutations in the FL-domain have a high potential for the development of serological DENV and ZIKV tests with high specificity.
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Keywords: dengue virus; Zika virus; diagnosis; cross-reactivity; ELISA; envelope proteins

INTRODUCTION

Arthropod-transmitted flaviviruses are small, enveloped RNA viruses, which are endemic to many parts of the world. They include a large number of important human pathogens, such as dengue, Zika, yellow fever, West Nile, Japanese encephalitis and tick-borne encephalitis viruses (DENV, ZIKV, YFV, WNV, JEV and TBEV, respectively).[1] On the basis of their antigenic properties, flaviviruses are divided into distinct serocomplexes, such as the JEV serocomplex (which contains JEV, WNV and others), or the DENV serocomplex (with the different dengue virus serotypes).[2] Among flaviviruses, DENV is causing the most severe impact on human health. Current estimates for DENV infections reach 400 million worldwide, occurring in over a hundred tropical and subtropical countries, and leading to thousands of deaths per year.[3] Recently, several candidate DENV-vaccines have been tested in clinical trials, and the first product was licensed in some endemic countries.[4,5] ZIKV, which had remained unnoticed in Africa for decades, emerged in the South Pacific in 2007 and was introduced to South America in 2014, where it is currently spreading.[6,7] ZIKV causes febrile illness, but it also appears to be linked to Guillain-Barré syndrome as well as microcephaly in newborns.[8] Many of the human pathogenic flaviviruses are transmitted by the same mosquito species (especially of the genus *Aedes*), and areas where different flaviviruses co-circulate are increasing in number, most importantly DENV and ZIKV in South America.[9,10]

Serological diagnosis of flavivirus infections relies on the measurement of IgM and IgG antibodies which usually appear about a week after symptoms onset.[11–13] Acute infections are diagnosed either by the detection of IgM or by a rising IgG antibody titer from the acute to the convalescent phases of infection. In secondary flavivirus infections, a measurable IgM response can be very low or absent [7,14] and IgG tests become the method of choice. However, specificity in flavivirus antibody-measurement is significantly hampered by the structural

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similarity of the viruses and the resulting antibody cross-reactivity.[15–17] This is of considerable concern especially for IgG-based assays, as it leads to false-positive test results.[18,19] The massive co-circulation of ZIKV and DENV has dramatically increased the problem. Antibodies against ZIKV are particularly cross-reactive to DENV and vice versa due to conserved parts of their proteins.[2] As a consequence, currently available tests are severely compromised by cross-reacting antibodies.[20] To validate serological results, virus neutralization tests have to be performed, which are time consuming and require BSL-3 facilities.[21] Therefore, there is a strong need for the development of techniques that have high sensitivity and specificity to detect and differentiate flavivirus antibodies and allow a high-throughput analysis.

The predominant target for cross-reacting antibodies is the fusion loop (FL) domain in the flavivirus envelope (E) protein, a short amino-acid sequence which is almost identical in many pathogenic flaviviruses.[22–24] It was shown before that mutating residues within this domain reduces binding of cross-reactive antibodies.[25–27] By using recombinant proteins that contain point mutations within and near the FL, we have previously generated systems to specifically diagnose WNV and DENV infections, respectively.[28,29] Here, we have analyzed the potential of this technology to differentiate antibody responses to DENV and ZIKV infections. Our results suggest that FL-mutant E-proteins of ZIKV and DENV can be used for a specific serological diagnosis of both infections.

MATERIAL AND METHODS

Human serum samples

Serum samples were divided into different groups regarding their virus infections, described in Tables 1 and 2. The origins of the samples were as follows:

DENV ($n=15$), ZIKV ($n=14$) and CHIKV ($n=8$) IgM and/or IgG-positive samples were obtained from persons returning from a stay in endemic areas, TBEV seropositive samples ($n=24$) were obtained from a seroprevalence study in forest rangers in Northeastern Italy, WNV seropositive sera ($n=28$) were obtained from patients with neuroinvasive disease or fever during outbreaks in Northeastern Italy. All these samples, as well as 14 negative control sera, were collected and characterized at Padova University Hospital, Italy. Ethical approval for these studies was obtained from the Padova University Hospital Ethics Committee. Another DENV ($n=31$), ZIKV ($n=18$) and JEV ($n=4$) IgM and/or IgG positive serum samples were obtained from returning travelers after a stay in endemic areas and were collected at the Bernhard Nocht Institute for Tropical Medicine, Hamburg, for diagnostic purposes with written informed consent from each patient. DENV PCR positive sera ($n=42$) were collected in Sri Lanka with the approval of Ethical Review Boards of the Medical Research Institute and Lady Ridgway Children Hospital Colombo. 13 ZIKV IgG positive sera were collected in Salvador, Brazil, from mothers having children with ZIKV infection-associated microcephaly. Sampling and testing were approved by the institutional research ethics board of the federal university of Bahia Climério de Oliveira. Another DENV ($n=14$) IgG and ZIKV ($n=9$) IgM and/or IgG positive samples were collected in Rio de Janeiro, Brazil, with ethical approval by the ethical committee from the University Hospital Clementino Fraga Filho (HUCFF). Additional DENV-positive samples ($n=14$), as well as one negative control, were obtained from Sera Care Life Sciences (Milford, CT, USA) and from Zeptometrix Corporation (Buffalo, NY, USA) (4 DENV-positive samples and two negative controls) with sample origin from Colombia, Honduras and Ecuador. The YFV ($n=8$) samples (obtained from the Robert Koch Institute, Berlin, Germany) were from YFV-vaccinated individuals who had participated in a randomized controlled vaccination study (approved by the national ethics committee). The Malaria immune sera ($n=6$) from Ghana were obtained from the Fraunhofer Institute for Molecular Biology and Applied Ecology, Aachen, Germany. Ethical clearance was obtained from the Committee on Human Research Publication and Ethics of the Kwame Nkrumah University of Science and Technology. All participants in this study provided informed consent and all samples were analyzed anonymously.

Antigens

The quadruple mutant E-protein from ZIKV (strain H/PF/2013, E-protein amino acid residues 1–406) bearing the point mutations T76A, Q77G, W101R and L107R was cloned into pMT/BiP/V5-His vector (Invitrogen, Carlsbad, CA, USA), expressed in *Drosophila S2* cells and purified from cell culture supernatants with IMAC and size exclusion chromatography as previously described for the DENV quadruple mutants,[29] which were generated accordingly. For

Table 1 Description of serum groups used for IgM measurements

Group	*N*	Characteristics	*N*	Origin	Year of sample collection	Diagnosis			
						PCR	Neutralization test	IgM ELISA	IgG ELISA
DENV	54	Acute sera from returning travelers and DENV endemic regions	12	Italy	2013–2016	10	4	12	7
			2	Zeptometrix		0	2	2	2
			4	Seracare (Colombia, Honduras, Equador)	2011	0	4	4	4
			17	Sri Lanka	2015	17	0	17	n.t.
			19	Germany	2011–2016	14	0	19	18
ZIKV	16	Acute sera from returning travelers and DENV endemic regions	2	Brazil	2015–2016	1	1	2	2
			8	Italy	2015–2016	6	2	8	7
			6	Germany	2016–2017	4	1	6	6
WNV	16	Residents in WNV endemic regions	16	Italy	2012–2013	0	16	16	16

Abbreviation: not tested, n.t.
Diagnosis was performed at the laboratories supplying the samples for this study, using commercial and in house assays.

Table 2 Description of serum groups used for IgG measurements

Group	Characteristics	Subgroup	*N*	Origin	Year of sample collection	Diagnosis			
						PCR	Neutralization test	IgM ELISA	IgG ELISA
DENV TL	Returning travelers	DENV TLa (Figure 3)	8	Italy	2013	4	6	5	8
		DENV TLb (Figure 5)	22	DENV TLa and Germany ($n=14$)	2011–2016	12	6	11	21
DENV END	Residents in DENV endemic regions	—	55	Zeptometrix ($n=2$)	2013	0	2	0	2
				Seracare (Colombia, Honduras, Equador) ($n=14$)	2011	0	14	4	14
				Brazil ($n=14$)	2008	0	8	0	14
				Sri Lanka ($n=25$)	2015	25	n.t.	n.t.	25
ZIKV TL	Returning travelers	ZIKV TLa (Figure 3)	12	Italy	2016	6	6	4	12
		ZIKV TLb (Figure 5)	19	ZIKV TLa and Germany ($n=7$)	2016–2017	9	7	4	19
ZIKV END	Residents in DENV endemic regions	—	21	Brazil	2015–2016	6	14	1	21 (19 also positive for DENV IgG)
WNV	Residents in WNV endemic regions	—	24	Italy	2012–2013	2	23	15	24
JEV	Returning travelers	—	4	Germany	2013–2016	0	n.t.	4	4
TBEV	Residents in TBEV endemic region	—	24	Italy	2013	0	n.t.	0	24
CHIKV	Returning travelers	—	8	Italy and Germany	2014–2015	0	0	7	8
YFV vac		—	8	Germany	2011–2013	n.t.	8	n.t.	8
Malaria		—	6	Ghana	2012	n.t.	n..t	n.t.	6

Abbreviation: not tested, n.t.
Diagnosis was performed at the laboratories supplying the samples for this study, using commercial and in house assays; n.t. = not tested.

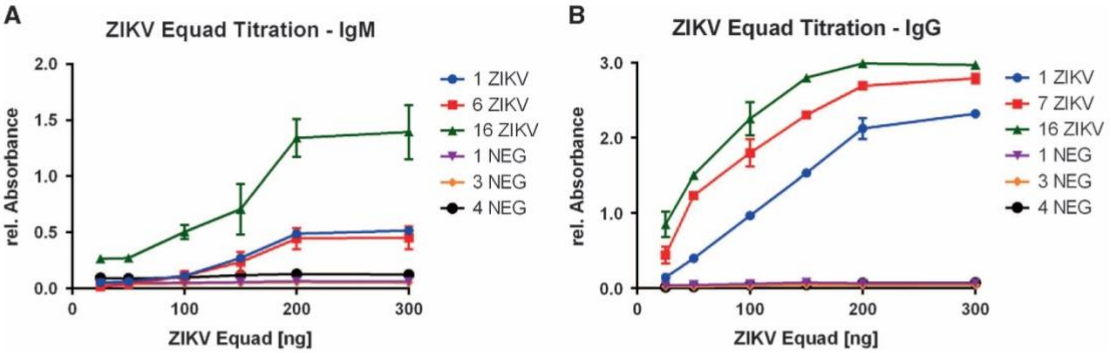

Figure 1 Titration curves of ZIKV Equad in an (**A**) IgM and (**B**) IgG ELISA with ZIKV- positive and negative sera.

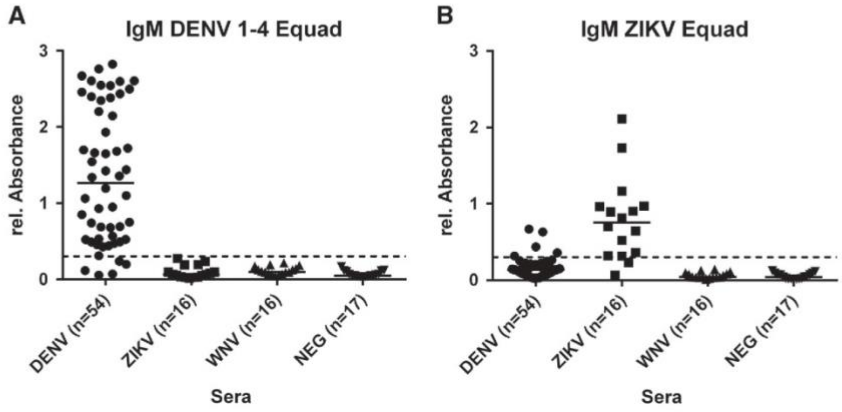

Figure 2 IgM ELISA on (**A**) 300 ng per well of DENV 1–4 Equad and (**B**) 200 ng per well of ZIKV Equad tested with IgM-positive DENV, ZIKV, WNV and flavivirus negative human sera. One sample per patient was examined in two independent experiments and plotted as a mean data point indicating absorbance values. The dotted lines represent cutoffs determined by a ROC analysis with negative sera as controls.

serological IgM and IgG assays, the four DENV 1–4 mutant antigens were mixed in ratios of 1:1:1:1 and 1:1:1:0.2 (due to increased cross-reactivity of DENV 4 Equad protein in IgG measurements, as described [29]), in concentrations of 300 ng and 160 ng per well, respectively, as described.[29] The ZIKV Equad antigen was used in the indicated amounts (Results section).

Antibody measurements

Indicated amounts of ZIKV Equad or DENV 1–4 Equad mixtures were coated overnight on Nunc polysorb plates (Thermo Scientific) in 100 µL coating buffer (15 mM Na2CO3, 35 mM NaHCO3, pH 9.6) at 4 °C. The plates were washed three times with 350 µL per well of PBS-0,05% Tween and blocked with 200 µL of 5% non-fat milk powder (blocking solution) for 2 h at room temperature. After a second washing step, human sera were diluted 1:100 in 100 µL blocking solution per well and incubated for 1.5 h at room temperature. Following a third washing step, the HRP-conjugated secondary goat anti human IgG (BioRad, Hercules, CA, USA, 1:10 000 in 100 µL blocking solution per well) or rabbit anti human µ-chain IgM (Dianova, 1:5000 in 100 µL blocking solution per well) antibody was added for 1 h at room temperature. After a fourth washing step, 100 µL TMB substrate (Biozol) per well were incubated for 30 min at room temperature. The reaction was stopped with 50 µL 1 M H_2SO_4 and signals were read out at 450 nm with background reduction at 520 nm in a micro plate reader (Infinite M200, Tecan).

In competition IgG ELISAs, sera (diluted 1:100 in blocking solution) were pre-incubated with indicated amounts of the competing antigen for 1 h at room temperature. Subsequently, they were added to the blocked antigens on the ELISA plate and incubated for 1.5 h at room temperature. The protocol was then continued as described above with IgG antibody detection.

Statistical analysis

All antibody measurements were performed in duplicates in at least two independent experiments, except in Figure 6, where single measurements were performed due to limited amounts of serum. Graphical and descriptive statistical analysis of data was carried out using GraphPad Prism 6 (La Jolla, CA, USA). Statistical significance was determined using the Holm-Sidak method, with alpha = 5.000%. Receiver operating characteristics (ROC) optimal curve calculations were performed in GraphPad Prism 6 with ELISA signals of the infected specimen and negative sera as control values. Signal cutoffs with optimal sensitivity and specificity were chosen and data were interpreted as positive with a signal/cutoff ratio higher than1.1 to ensure the best specificity. The cutoffs for the individual assay types are listed in Supplementary Table S1.

RESULTS

To facilitate a specific serological differentiation between DENV and ZIKV infections, we inserted four amino acid point mutations in the

conserved fusion loop (FL) domain of the ZIKV E-protein (Equad) and compared it to the previously described DENV 1–4 Equad mixture, which was shown to significantly reduce cross-reactivities in dengue serological diagnosis.[29]

The optimal concentration of ZIKV Equad for ELISA-based IgG and IgM tests was established through titration of the antigen with three ZIKV-positive and -negative sera each (Figure 1). Saturation of ZIKV-positive signals was observed at 200 ng per well in IgM- and IgG-measurements. Negative sera did not show any background in both setups through all tested antigen amounts, indicating a high specificity of the purified ZIKV Equad.

For the DENV 1–4 Equad mixture, the antigen amount per well yielding optimal sensitivity and specificity in IgM- and IgG- based ELISAs was determined previously.[29]

IgM antibodies of DENV-, ZIKV- and WNV-infected human sera (Table 1) and negative control sera were measured on the DENV (1–4) Equad mixture (Figure 2A) and on ZIKV Equad (Figure 2B). The sera from DENV- and ZIKV- infected individuals showed specific binding on DENV 1–4 Equad and ZIKV Equad, respectively. Negative control sera were used to calculate a ROC cutoff for each antigen (Supplementary Table S1) resulting in 90.7% sensitivity for DENV Equad and 87.5% sensitivity for ZIKV Equad. Both antigens showed 100% specificity of infected sera in comparison to the flavivirus-negative control samples (Table 3, Supplementary Table S2). No cross-reactivity was observed on DENV 1–4 Equad with ZIKV- and WNV-IgM-positive sera, resulting in 100% specificity of this assay (Figure 2A). The ZIKV Equad antigen yielded 5/54 cross-reactive DENV- infected samples reducing its specificity versus DENV to 90.7%(total specificity of 94.3% with all ZIKV-negative control samples). A statistical analysis of all measurements described is shown in Supplementary Tables S2–S4.

Next, we used the DENV 1–4 Equad mixture and ZIKV Equad to measure IgG responses in serum samples from DENV-, ZIKV-, WNV-, JEV- and TBEV-infected persons (Figure 3). For DENV and ZIKV, sera derived from European travelers returning from endemic areas (groups DENV-TLa, ZIKV-TLa, Table 2) were used, which represent mostly primary infections. Non-infected individuals as well as samples from CHIKV- and Malaria-infected and YFV-vaccinated persons served as controls. Sera of non-infected individuals were used for ROC analysis and cutoff calculation in comparison to the DENV- and ZIKV-positive serum samples resulting in 100% sensitivity for both antigens (Table 3, Supplementary Table S1). Sera from WNV-, JEV-, TBEV-, CHIKV-, Malaria-infected and YF-vaccinated individuals showed no cross-reactivity on the DENV- and ZIKV- Equad antigens and were detected as negative according to the ROC cutoffs. For DENV- and ZIKV- positive sera, a statistically significant overall signal reduction was observed when DENV-positive specimen were measured on ZIKV Equad and ZIKV-positive sera on the DENV Equad in comparison to signals on the homologous antigen (Supplementary Table S3). However, when using the ROC cutoff, both groups displayed cross-reactive signals leading to several false-

Table 3 Sensitivity and specificity of DENV- and ZIKV- Equad in IgM- and IgG- measurements

	DENV Equad						ZIKV Equad				
Sera	**N**	**Positive**	**Sensitivity**	**Specificity**	**95% CI**	**Sera**	**N**	**Positive**	**Sensitivity**	**Specificity**	**95% CI**
IgM											
DENV	**54**	**49**	90.74%	—	79.70%–96.92%	DENV	54	5		90.74%	79.70%–96.92%
ZIKV	16	0	—	100%	79.41%–100%	**ZIKV**	**16**	**14**	87.5%	—	61.65%–98.45%
WNV	16	0	—	100%	79.41%–100%	WNV	16	0		100%	79.41%–100%
NEG	17	0	—	100%	80.49%–100%	NEG	17	0		100%	80.49%–100%
Total control sera	49	0		**100.00**	92.75%–100%	Total control sera	87	5		**94.25%**	84.12%–96.70%
IgG											
DENV Tla	**9**	**9**	100%	—	63.37%–100%	DENV TLa	9	5	—	44.44%	10.70%–48.41%
ZIKV Tla	12	5	—	58.33%	18.44%–67.08%	**ZIKV TLa**	**12**	**12**	100%	—	15.70%–84.30%
WNV	24	0	—	100%	85.75%–100%	WNV	24	0		100%	85.75%–100%
JEV	4	0		100%	39.76%–100%	JEV	4	0		100%	39.76%–100%
TBEV	24	0	—	100%	85.75%–100%	TBEV	24	0		100%	85.75%–100%
CHIKV	8	0	—	100%	63.06%–100%	CHIKV	8	0		100%	63.06%–100%
YFVvac	8	0	—	100%	63.06%–100%	YFVvac	8	0		100%	63.06%–100%
MAL	6	0	—	100%	54.07%–100%	MAL	6	0		100%	54.07%–100%
NEG	17	0	—	100%	80.49%–100%	NEG	17	0		100%	80.49%–100%
Total control sera	103	5		**95.15%**	89.03%–98.41%	Total control sera	100	5		**95%**	88.72%–98.36%
IgGcomp											
DENV TLb	**23**	**23**	100%	—	85.18%–100%	DENV TLb	23	0	—	100%	85.18%–100%
DENV END	**55**	**55**	100%	—	93.51%–100%	DENV END	55	2	—	96.36%	87.47%–99.56%
ZIKV TLb	18	1	—	94.44%	73.97%–99.87%	**ZIKV**	**18**	**18**	100%	—	81.47%–100%
(ZIKV END)[a]	21	18	—	14.29%	3.05%–36.34%	**ZIKV END**	**21**	**21**	100%	—	83.89%–100%
NEG	17	0	—	100%	80.49%–100%	NEG	17	0	0	100%	80.49%–100%
Total control sera	35	1		**97.14%**	85.08%–99.93%	Total control sera	95	2		**97.89%**	92.60%–99.74%

Abbreviation: Confidence interval, CI, refers to the number of samples in the cohort. Bold entries represent sera groups which are infected with a to the antigen homologous virus and also total specificites of each test are highlighted in bold.
[a]Group was excluded from specificity measurements because of 90% DENV seroprevalence.

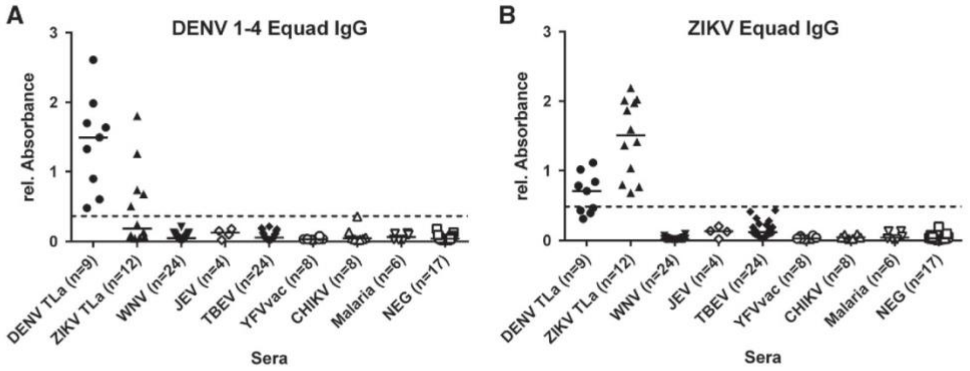

Figure 3 IgG ELISA on (**A**) 160 ng per well of DENV 1–4 Equad and (**B**) 150 ng per well of ZIKV Equad measured with flavivirus positive sera, CHIKV and Malaria positive and negative sera. One sample per patient was examined in two independent experiments and plotted as a mean data point indicating absorbance values. The dashed lines represent cut-offs determined by a ROC analysis with negative sera as controls.

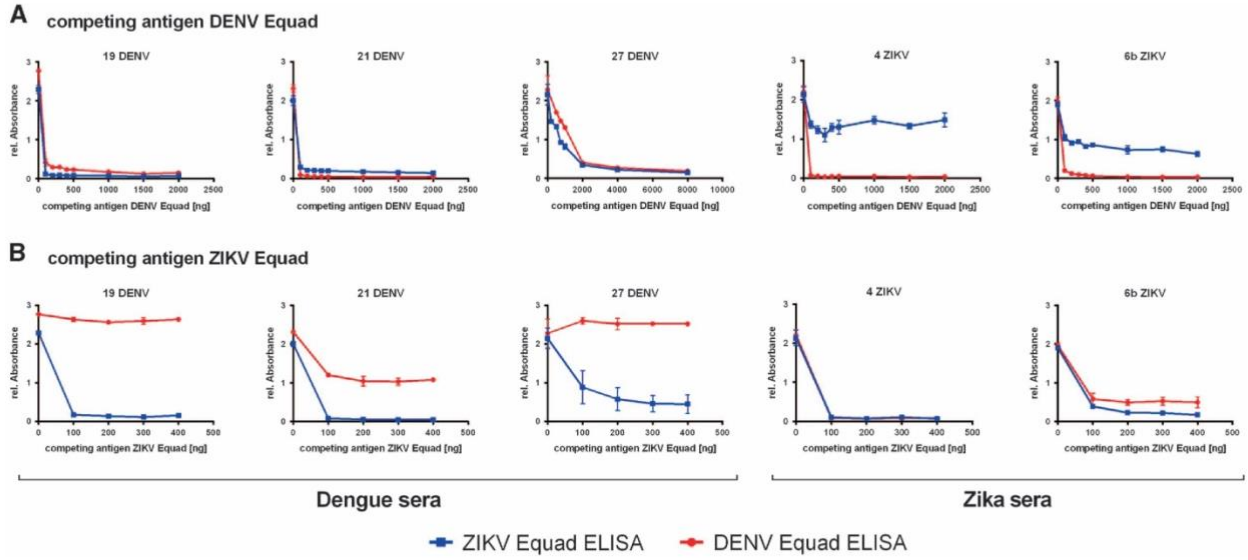

Figure 4 Titration of the competing antigen (**A**) DENV Equad and (**B**) ZIKV Equad in Dengue and Zika positive sera and IgG measurement on the coated DENV Equad (blue lines) and ZIKV Equad (red lines).

positive results of ZIKV-infected individuals in the DENV- IgG-ELISA and of DENV-infected individuals in the ZIKV-IgG-ELISA.

To further reduce the IgG cross-reactivity between DENV- and ZIKV- positive sera, a competition ELISA setup was chosen and tested with 3 DENV-positive sera which were known to be ZIKV-negative because they were collected in South America before the ZIKV-outbreak and 2 ZIKV-infected samples. All of them displayed similarly high signals in both the DENV- and the ZIKV- IgG ELISA. These sera were now pre-incubated with a competing antigen (either ZIKV Equad or the DENV 1–4 Equad mixture) and then measured on the coated DENV 1–4 Equad or ZIKV Equad. Figure 4A displays competition experiments with DENV 1–4 Equad that resulted in a signal drop for dengue positive sera on both antigens caused by the removal of DENV-specific and ZIKV-cross-reactive antibodies with increasing amounts of DENV 1–4 Equad as competing antigen. In the DENV-positive serum 27, removal of DENV- and cross-reactive ZIKV-antibodies was seen only with 2 µg competitor antigen, about 10fold more than in the other samples. Competition of ZIKV-positive sera with DENV 1–4 Equad also strongly decreased the signals in DENV-ELISAs, but only slightly influenced the signals on ZIKV Equad through all tested competing antigen amounts. The competition with at least 200 ng of ZIKV Equad (Figure 4B) eliminated ZIKV cross-reactive antibodies in DENV-infected sera, which then showed low signals in ZIKV-ELISAs and still well detectable signal intensities in DENV-ELISAs. For one specimen (21DENV) a signal drop was observed in DENV-ELISA with ZIKV-competition (Figure 4B), similar to the ZIKV-infected samples with DENV-competition in the ZIKV-ELISA (Figure 4A). This illustrates the varying amounts of cross-reactive antibodies in individual serum samples. The two ZIKV-positive sera that were competed with ZIKV Equad showed a signal drop with 200 ng competitor in both ZIKV- and DENV-ELISAs (Figure 4B).

After having established suitable conditions for competition experiments, DENV- and ZIKV- IgG-positive as well as the negative sera were then measured on DENV 1–4 Equad after competition with 0.2 µg ZIKV Equad (Figure 5A) and on ZIKV Equad after competition

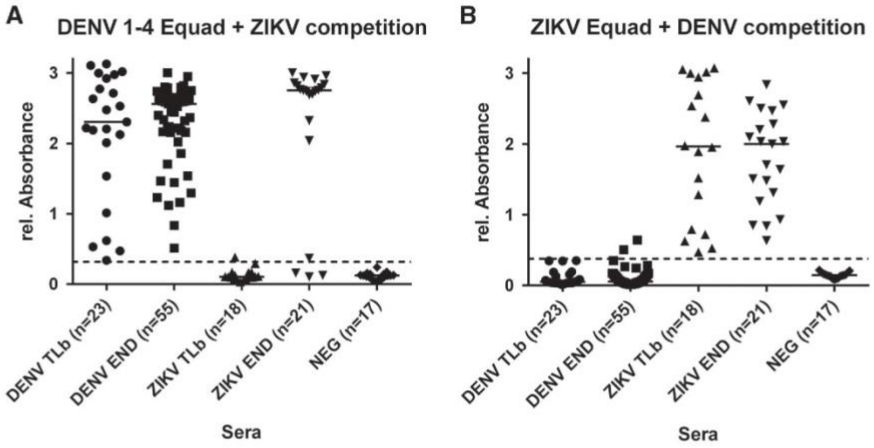

Figure 5 IgG Competition ELISA: Sera were measured on (**A**) 160 ng/well of DENV 1–4 Equad with 200 ng per well ZIKV Equad competition and (**B**) 150 ng of ZIKV Equad with 2 µg of DENV 1–4 competition. One sample per patient was examined in two independent experiments and plotted as a mean data point. The dashed lines represent cutoffs determined by a ROC analysis with negative sera as controls.

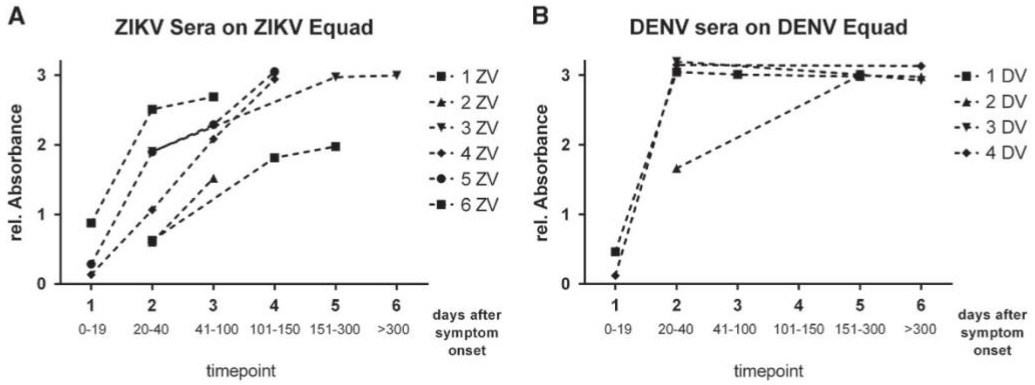

Figure 6 IgG competition ELISA of paired serum samples from European travelers infected with ZIKV (**A**) and DENV (**B**) measured on 150 ng of ZIKV Equad (**A**) with DENV Equad competition and on 160 ng of DENV 1–4 Equad (**B**) with ZIKV competition.

with 2 µg of DENV 1–4 Equad (Figure 5B). The higher amount of the DENV antigen as a competitor was chosen in order to correctly analyze sera such as sample 27DENV (Figure 4A), which only showed effective competition after incubation with 2 µg of DENV 1–4 Equad.

ZIKV- and DENV-positive sera were divided into two different groups each (Table 2): European travelers returning from endemic countries (primary infections, DENV-TLb and ZIKV-TLb) and patients from DENV endemic areas in Brazil and Sri Lanka (DENV-END). A co-infection with ZIKV in the DENV-END group could be excluded because Brazilian samples were taken in 2008, before the ZIKV outbreak, and Sri Lanka samples were from 2015 with no known circulation of ZIKV. However, secondary DENV-infections were included in this group. In contrast, the samples in the ZIKV-END group all derived from DENV-endemic areas in Brazil and were taken in 2016. A previous DENV-infection is therefore likely and was also found previously with serological test methods in 90% of those samples (Table 2). In comparison to the IgG assay without competition, the specificity of both the DENV and the ZIKV Equad ELISAs (Figure 5 and Supplementary Table S4) was strongly enhanced (100% for ZIKV Equad and 94.4% for DENV Equad) in the DENV-TLb and ZIKV-TLb groups. Also in the DENV-END group, all serum samples were detected as positive for DENV and only two signals were over the cutoff on the ZIKV antigen. The ZIKV-END samples were all detected as positive for ZIKV (100% sensitivity), but 85.7% of the samples also reacted in the DENV-ELISA, which correlates well to the prior DENV infections present in this group. The course of IgG production against ZIKV and DENV was also determined using paired samples from the returning traveler groups. As can be seen from Figure 6, antibody levels increased during the acute phase in the first 40 days after symptom onset and remained stable for up to 300 days.

DISCUSSION

Cross-reactivity is a long-known complication in the serological diagnosis of flavivirus-infections, and several attempts have been made to develop systems to increase specificities of available test systems.[25,26,28–30] However, due to the epidemic spread of ZIKV in areas of simultaneous DENV circulation, the problem has gained another dimension. The specific diagnosis of these infections is mandatory not only for the implementation of effective control and surveillance activities, including the conduction of recently started DENV vaccine trials, but also for the timely treatment of potentially life-threatening disease symptoms. However, available DENV-tests are

severely affected by ZIKV-antibodies, leading to a large number of false positive test results.[20,31,32] Recently, ZIKV ELISAs based on NS1-proteins as antigens have been made commercially available, but limitations in IgM and/or IgG detection have been reported.[33–36] Here we present a system for the specific and sensitive serological diagnosis of DENV- and ZIKV infections, based on the E-protein as antigen. The E-protein is widely used in serological flavivirus diagnosis, as virtually all infected individuals generate antibodies against it. On the other hand, it is targeted by cross-reactive antibodies, which mainly recognize the conserved FL-domain. By using mutations within and next to the FL-domain of the E-proteins of DENV and ZIKV, we found that IgM antibodies show greatly reduced cross-reactivity and bind specifically to the homologous antigen. This is in line with previous reports indicating that IgM responses against flaviviruses are more specific than IgG,[19] although cross-reactivity has also been described.[7,37] However, IgM antibodies are often produced only in low amounts, especially in secondary flavivirus infections or after a vaccination and are shortlived,[14,38] hence IgG measurements become necessary. Using the mutant E-proteins, IgG antibodies present in the DENV- and ZIKV-positive sera did show cross-reactive binding to the ZIKV- and the DENV-antigens, respectively, although serum samples from WNV-, JEV- and TBEV-infected persons remained negative in both ELISAs, confirming previous results with the DENV antigen.[29] On the basis of the sequence conservation of the E-protein ectodomains, ZIKV is more closely related to DENV than to the JEV-serocomplex, with amino acids identities of 52% and 54–57% when comparing ZIKV to WNV and DENV (four serotypes), respectively.[2,39] This minor difference in amino acids identities is apparently enough to result in a very different binding of cross-reactive antibodies in sera from infected persons. Antibodies against non-flaviviral vector-borne pathogens (malaria, chikungunya) did not bind to either antigen, which underlines the usability of the mutant E-proteins in the diagnosis of flavivirus infections, whereas false-positive results with sera from malaria infected persons have been reported for an NS1-based ZIKV assay.[40,41]

In order to increase the specificity of the test in the differentiation of DENV- from ZIKV IgG-antibodies and vice versa, we performed competition experiments by pre-incubating the sera with one antigen before measuring them on the ELISA plate coated with the second one. This resulted in a statistically significant reduction of cross-reactivities (Supplementary Table S3). Now, ZIKV-infected samples from returning travelers did not show signals in the DENV ELISA, and the same was the case for DENV-infected returning travelers in the ZIKV-test. This demonstrates that, after the competition step, the mutant antigens are able to differentiate DENV- and ZIKV- IgG responses. When analyzing DENV-positive samples from inhabitants of DENV-endemic areas, these were detected as DENV-positive with high specificity and sensitivity and only 2/55 displayed low signals over the cutoff in the ZIKV ELISA. Whereas DENV secondary infections can be assumed for many of the samples within this group, ZIKV-infections can be excluded. The ZIKV-positive serum samples in the ZIKV-END group derived from areas with a high DENV-seroprevalence that can reach up to 80%,[42] which corresponds to the detection of DENV-antibodies in 18/21 samples using the mutant antigens. This result underlines the need to simultaneously test for ZIKV- and DENV- IgG antibodies in areas of co-circulation.

Such secondary flavivirus infections represent a major challenge, not only for serological diagnosis. Pre-existing flavivirus antibodies might lead to severe complications of acute DENV- or ZIKV-infections, including severe dengue and neurological or congenital complications with ZIKV, as has been suggested by several studies.[43–45] Therefore, tests to identify and discriminate such antibodies are an unmet need which has to be addressed urgently. Methods as the one presented here, which are able to detect DENV and ZIKV IgM- and IgG-antibodies with high specificity and sensitivity, could be very useful in this respect.

Using paired serum samples we analyzed the rise of IgG antibodies upon infection with ZIKV and DENV in returning European travelers. A strong increase in the first 40 days was observed, and DENV-antibodies seemed to reach a plateau earlier than ZIKV antibodies, which might be attributed to a generally higher viral load in infections with DENV compared to ZIKV.[46] However, higher numbers of serum samples need to be analyzed in order to test whether this is indeed a difference between the two infections

Most of the currently available ZIKV-ELISAs are based on the NS1 protein. Recently published attempts for improvement of ZIKV serological diagnosis include a microarray-based assay using ZIKV NS1 and DENV virus particles in a multiplexed format based on a plasmonic gold platform.[47] and a monoclonal ZIKV NS1-antibody in a blockage-of-binding format.[48] The system presented here relies on another antigen (mutant E-protein) and could, therefore, be used as an alternative approach in serology, independent of NS1 and also not dependent on only a single epitope. As the infrastructure for virus neutralization tests is only available in few specialized laboratories, confirmation and evaluation of inconclusive results from a particular serological assay can be performed best with an assay that is based on a different antigen. Therefore, resulting ELISAs for ZIKV and DENV could contribute to an accurate diagnosis and surveillance of these two virus infections, both as stand-alone tests and as means to complement existing methods.

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Supplementary Information for this article can be found on the *Emerging Microbes and Infections* website (http://www.nature.com/emi)

3 Zusammenfassung

Dissertation zur Erlangung des akademischen Grades:
Dr. rer. nat.

Titel:
Entwicklung von Verfahren für die spezifische, serologische Diagnostik von Dengue- und Zika-Virusinfektionen mit modifizierten *Envelope* Proteinen

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Das Dengue-Virus ist ein, in tropischen und subtropischen Regionen endemisches, humanpathogenes Virus, welches durch Moskitos des Genus *Aedes* übertragen wird. Jährlich werden ca. 95 Millionen der Infektionen klinisch manifestiert, wobei 1 % davon in der schwersten Form des Dengue-hämorrhagischen Fiebers verlaufen. Eine schnelle und einfache Diagnostik spielt dabei für die Behandlung sowie für epidemiologische Studien eine wichtige Rolle. Die serologische Diagnostik flaviviraler Infektionen im Allgemeinen und DENV im Speziellen, ist durch die Vielzahl kreuzreaktiver und infektionsverstärkender Antikörper hoch komplex. Die starke Co-Zirkulation verschiedener Flaviviren und deren ähnliche klinische Symptomatik erschwert zusätzlich die spezifische Diagnostik. Seit 2015 führte insbesondere der massive Vormarsch von ZIKV-Infektionen in DENV-endemischen Gebieten Südamerikas zu einer noch komplexeren Sachlage, da die serologische Unterscheidung beider Infektionen kaum möglich war.

Im Rahmen dieser Promotionsarbeit wurden daher rekombinante virale E-Proteine exprimiert und charakterisiert, welche die Spezifität serologischer DENV- und ZIKV-Tests erhöhen sollen. E-Proteine sind stark immunogen, da sie die Virusoberfläche dominieren. Jedoch ist deren hochkonservierte DII-FL Region gleichzeitig das Ziel von einem Großteil kreuzreaktiver Antikörper. Studien mit WNV und JEV haben zuvor belegt, dass Mutationen in diesem Sequenzbereich, die Kreuzreaktivität mit heterologen Flaviviren herabsenken können. Deshalb wurden vier Punktmutationen in die DII-FL Region von DENV 1-4 und ZIKV E (Equad) eingefügt und in einem Insektentenzellen- basierten System exprimiert, aus dem Kulturüberstand aufgereinigt und mit Humanseren in IgM- und IgG-ELISAs untersucht. Die Ergebnisse dieser Arbeit werden in zwei Publikationen dargelegt, welche die Grundlage für diese Promotionsschrift darstellen. Im Folgenden werden die wichtigsten Resultate in Thesenform zusammengefasst.

I. In der ersten Publikation werden zunächst die mutierten DENV 1-4 Equad Proteine im Vergleich zu DENV-2 E wildtyp (Ewt) exprimiert, charakterisiert und in Hinsicht auf deren Vermögen, die Spezifität in IgM- und IgG-ELISAs mit heterologen flaviviralen Seren zu erhöhen, untersucht.

   1. Die DENV 1-4 Equad, sowie DENV-2 Ewt Proteine können erfolgreich in *Drosophila* S2 Zellen exprimiert und aus dem Kulturüberstand aufgereinigt werden. Die Aufreinigung erfolgt zweistufig durch eine Nickel-Affinitätschromatographie mit einer anschließenden Entfernung unspezifischer Bindungspartner durch eine Größenausschlusschromatographie.

   2. Die allgemeine Strukturintegrität in der DI/DII und DIII Domäne bleibt, trotz eingefügter Mutationen in der DII-FL Region, erhalten. Die Charakterisierung der Strukturintegrität erfolgt exemplarisch mit DENV-2 Equad und Ewt durch Bindung monoklonaler Antikörper, welche strukturelle Domänen des DENV E-Proteins erkennen.

   3. Der direkte Vergleich von je 300 ng/well DENV-2 Equad und Ewt zeigt in IgG-ELISAs mit WNV- und TBEV-Seren eine starke Kreuzreaktion auf dem Ewt Protein und deren signifikante Abnahme auf dem mutanten Equad. Gleichzeitig nimmt der Median der Signalintensität der getesteten DENV-Seren auf dem Equad Protein im Vergleich zum Ewt geringfügig ab. Gelbfieber-geimpfte und Flavivirus-negative Humanseren zeigen auf beiden Antigenen eine vernachlässigbare Bindung und verdeutlichen die Reinheit der hergestellten Proteine.

4. Durch Titrationskurven der DENV 1-4 Equad Proteine kann ein optimales Mischungsverhältnis der Serotypen 1-4 von 1:1:1:0,2 ermittelt werden, welches im IgG-ELISA die Sensitivität mit DENV-positiven Seren, bei gleichzeitig herabgesetzter Antigenmenge, erhöht. Weiterhin wird so die Spezifität gegenüber den WNV- und TBEV IgG positiven Humanseren nochmals gesteigert, wodurch deren klare Abgrenzung zu den IgG-Signalen von DENV-infizierten Seren erreicht wird. Vergleichend wird ein Teil der Seren in einem kommerziell erhältlichen DENV IgG-ELISA getestet, wobei dort 100 % der WNV- und 30 % der TBEV Seren falsch positiv detektiert werden.

5. Im IgM-ELISA wird im Vergleich zu IgG-Messungen eine niedrigere Kreuzreaktivität von IgM positiven WNV-Seren auf 300 ng/well DENV-2 Ewt beobachtet. Die Messung dieser mit der gleichen Gesamtmenge von DENV 1-4 Equad in einem Verhältnis von 1:1:1:1 zeigt die signifikante Reduzierung der Kreuzreaktionen und die gleichzeitige Erhöhung der Sensitivität von IgM positiven DENV-Seren. Auch im IgM-ELISA zeigen negative Referenzseren sowohl mit DENV 1-4 Equad als auch DENV-2 Ewt kein Hintergrundsignal.

II. In der zweiten Publikation erfolgt die Ausweitung des Equad Systems auf die serologische Diagnostik von ZIKV-Infektionen und deren Differenzierung zu Infektionen mit DENV, weshalb die bestehenden DENV 1-4 Equad IgM- und IgG-ELISAs im Hinblick auf die Spezifität gegenüber ZIKV positiver Humanseren re-evaluiert werden.

1. Die Expression und Aufreinigung von ZIKV Equad erfolgt, analog zum bestehenden System mit DENV 1-4 Equad, in *Drosophila S2* Zellen mit der anschließenden zweistufigen Chromatographie des Zellkulturüberstandes durch Nickel-Affinität und Größenausschluss.

2. Die optimale ZIKV Equad Konzentration für IgM- und IgG-ELISAs wird durch Titrationskurven mit ZIKV positiven und negativen Humanseren bestimmt. Sie beträgt für IgM-ELISAs 200 ng/well und für IgG-Messungen 150 ng/well.

3. In IgM-ELISAs werden DENV-, ZIKV- und WNV-positive Seren auf DENV 1-4 und ZIKV Equad getestet, sowie ROC Cut-offs mit negativen Referenzseren bestimmt. Für DENV 1-4 ergibt sich dabei eine Spezifität von 100 % und eine Sensitivität von 91 %. Auf dem ZIKV Equad werden wenige Kreuzreaktionen von DENV-Seren verzeichnet, wodurch die Spezifität dieses Tests 94 % und die Sensitivität 88 % beträgt

4. IgG-ELISAs mit DENV 1-4 Equad und ZIKV Equad zeigen mit WNV-, JEV-, TBEV-, CHIKV-, Malaria infizierten und YFV-geimpften Humanseren keine Kreuzreaktionen und eine 100%ige Sensitivität mit den, zum Antigen homologen, DENV- und ZIKV-Seren. Hingegen wird eine starke Kreuzreaktion von primärinfizierten DENV- und ZIKV- Seren auf das jeweils heterologe Antigen gemessen, die in geringen Spezifitäten von 58 % im DENV-ELISA und 44 % im ZIKV-ELISA resultieren.

5. Für die ZIKV- und DENV-IgG-ELISAs wird zur Steigerung der Spezifität ein Kompetitionsschritt mit dem jeweils heterologen Equad Antigen entwickelt, welches die kreuzreaktiven Antikörper in den Seren bindet.

6. In kompetitiven IgG-ELISAs erhöhen sich die Spezifitäten der ZIKV- und DENV-Equad Antigene jeweils auf 97,9 % und 97,1 %. Im Speziellen beträgt die Spezifität des ZIKV-ELISAs gegenüber primär infizierter DENV-Seren von Reiserückkehrern 100 % und ergibt 96 % gegenüber der Kontrollgruppe bestehend aus DENV-positiven Seren von Einwohnern DENV-endemischer Regionen, bei denen DENV-Sekundärinfektionen inkludiert wurden, jedoch ZIKV Infektionen aufgrund der geographischen und zeitlichen Einordnung ausgeschlossen werden konnten. Das DENV 1-4 Equad Antigen zeigt eine Spezifität von 94 % gegenüber Seren ZIKV-infizierter Reiserückkehrer. ZIKV-positive Seren aus DENV-endemischen Regionen Brasiliens reagieren zum Großteil auch positiv im DENV-ELISA und resultieren in einer Spezifität von 14 %. Dieses Ergebnis stimmt jedoch mit der hohen DENV-Seroprävalenz dieser Gebiete überein. Die Sensitivität beider ELISAs beträgt 100 %.

Abschließend lässt sich zusammenfassen, dass die Insertion von vier Punktmutationen in der DII-FL Region von DENV- und ZIKV- E-Proteinen zu einer signifikant erhöhten Spezifität in der Detektion von DENV- und ZIKV-induzierten IgM- und IgG-Antikörpern führt. Eine Unterscheidung beider Infektionen auf IgG-Ebene kann dabei, aufgrund der hohen Sequenz- und Strukturhomologie von DENV- und ZIKV- E-Proteinen, durch einen kompetitiven ELISA erreicht werden. Die, in dieser Promotionsarbeit dargelegten, Resultate leisten daher einen wichtigen Beitrag für die Entwicklung serologischer Verfahren zur spezifischen Diagnose und Überwachung von DENV- und ZIKV-Infektionen.

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Anlagen (Supplementary information)

Anlagen zur Publikation Nr.1

Supplementary table 1: Analysis of the DENV E proteins with monoclonal antibodies

mAb	DENV-2 Ewt	DENV-2 Equad	DENV-1 Equad	DENV-3 Equad	DENV-4 Equad
DV2-76	1.413	1.111	-	-	-
DV2-96	1.126	1.052	-	-	-
DV2-106	0.880	0.706	-	-	-
DV2-44	0.980	0.726	-	-	-
DV2-29	1.688	0.154	-	-	-
WNV E18	2.267	0.149	0.188	0.300	0.172
WNV E60	1.690	0.174	0.270	0.343	0.249

Supplementary table 1: analysis of the DENV E proteins with monoclonal antibodies (mAb) recognizing conformational epitopes. 2 mg/ml of protein were coated onto 96-well plates and an ELISA was performed. Values indicate absorbance (450nm) and are from one representative experiment of two.

Antibodies:

DENV-2 specific: DV2-44 (domains DI-DII); DV2-76, DV2-96, DV2-106 (domain DIII); DV2-29 (FL-domain)

WNV-specific/cross reactive: E18, E60 (FL-domain).

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Supplementary table 2: statistical analysis

Figure 4A

sera	*mean*	*STDV*
DENV	2.364	0.480
WNV	1.261	0.594
TBEV	0.821	0.588
YFV	0.149	0.081
NEG	0.218	0.217

Figure 4B

sera	*mean*	*STDV*	*difference to DENV-2 Ewt §*
DENV	1.896	0.702	**
WNV	0.106	0.059	**
TBEV	0.160	0.138	**
YFV	0.033	0.01	**
NEG	0.075	0.033	NS

Figure 4C

sera	*mean*	*STDV*	*difference to DENV-2 Ewt §*	*difference to DENV-2 Equad §*
DENV	1.816	0.549	**	NS
WNV	0.063	0.031	**	*
TBEV	0.084	0.058	**	NS
YFV	0.047	0.018	*	NS
NEG	0.052	0.023	*	NS

Figure 5A

sera	*mean*	*STDV*
DENV	0.852	0.388
WNV	0.265	0.189
NEG	0.072	0.095

Figure 5B

sera	*mean*	*STDV*	*difference to DENV-2 Ewt §*
DENV	1.249	0.691	*
WNV	0.112	0.059	*
NEG	0.067	0.062	NS

§: Mann-Whitney Rank Sum Test. The Two asterisks (**) indicate statistical significance with

P < 0,001; one asterisk (*) indicates P < 0,05. NS: not significant.

Anlagen zur Publikation Nr.2

Supplementary Table S1: ROC positivity cut-offs of DENV- and ZIKV- Equad for IgG- and IgM- measurements. Cut-offs were calculated with negative sera (N=17) as controls to achieve optimal specificity and sensitivity

Detection	Antigen	ROC cut-off	Area under the ROC Curve (95% CI)
IgM	DENV Equad	> 0.2732	0.9826 (0.9586 – 1.007)
	ZIKV Equad	> 0.2734	0.9743 (0.9213 – 1.027)
IgG	DENV Equad	> 0.3323	1.0 (1.0)
	ZIKV Equad	> 0.4425	1.0 (1.0)
	DENV Equad + ZIKV comp	> 0.2899	1.0 (1.0)
	ZIKV Equad + DENV comp	> 0.3437	1.0 (1.0)

CI: confidence interval

Supplementary Table S2: Statistical analysis of IgM results with Sidak's multiple comparison test (DENV Equad vs ZIKV Equad); asterisks indicate significant results (p-values are shown)

Group	Mean on DENV Equad	Mean on ZIKV Equad	Mean diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
DENV (n=54)	1.350	0.1580	1.192	0.9564 to 1.428	Yes	****	< 0.0001
ZIKV (n=16)	0.0943	0.7948	-0.7005	-1.133 to -0.2677	Yes	***	0.0003
WNV (n=16)	0.1140	0.06178	0.05222	-0.3806 to 0.4851	No	ns	0.9968
NEG (n=17)	0.0665	0.05121	0.01529	-0.4046 to 0.4352	No	ns	> 0.9999

Supplementary Table S3: Statistical analysis of IgG Results with Sidak's multiple comparison test (DENV Equad vs ZIKV Equad); asterisks indicate significant results (p-values are shown)

Group	Mean on DENV Equad	Mean on ZIKV Equad	Mean diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
DENV TLa (n=9)	1.415	0.6747	0.7403	0.4246 to 1.056	Yes	****	< 0.0001
ZIKV TLa (n=12)	0.4730	1.479	-1.006	-1.279 to -0.7326	Yes	****	< 0.0001
WNV (n=24)	0.06401	0.02456	0.03945	-0.1539 to 0.2328	No	ns	0.9995
TBEV (n=24)	0.08097	0.1575	-0.07653	-0.2699 to 0.1168	No	ns	0.9410
YFVvac (n=8)	0.03643	0.0350	0.001430	-0.3334 to 0.3363	No	ns	> 0.9999
JEV (n=4)	0.1130	0.1226	-0.009600	-0.4832 to 0.4640	No	ns	> 0.9999
CHIKV (n=8)	0.08903	0.04823	0.0408	-0.2941 to 0.3757	No	ns	> 0.9999
Malaria (n=6)	0.06712	0.06261	0.00451	-0.3822 to 0.3912	No	ns	> 0.9999
NEG (n=17)	0.0548	0.04546	0.009340	-0.2204 to 0.2391	No	ns	> 0.9999

Supplementary Table S4: Statistical analysis of IgG competition results with Sidak's multiple comparison test (DENV Equad vs ZIKV Equad); asterisks indicate significant results (p-values are shown)

Group	Mean on DENV Equad	Mean on ZIKV Equad	Mean diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
DENV TLb (n=23)	2.124	0.1040	2.020	1.583 to 2.457	Yes	****	< 0.0001
DENV END (n=55)	2.294	0.1034	2.191	1.908 to 2.473	Yes	****	< 0.0001
ZIKV TLb (n=18)	0.1305	1.915	-1.785	-2.278 to -1.291	Yes	****	< 0.0001
ZIKV END (n=21)	2.257	1.797	0.4600	0.003091 to 0.9169	Yes	*	0.0476
NEG (n=17)	0.1196	0.1455	-0.02590	-0.5337 to 0.4819	No	ns	> 0.9999

Erklärung über die eigenständige Abfassung der Arbeit

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar eine Vergütung oder geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt an der Entstehung der vorliegenden Arbeit beteiligt waren. Die aktuellen gesetzlichen Vorgaben in Bezug auf die Zulassung der klinischen Studien, die Bestimmungen des Tierschutzgesetzes, die Bestimmungen des Gentechnikgesetzes und die allgemeinen Datenschutzbestimmungen wurden eingehalten. Ich versichere, dass ich die Regelungen der Satzung der Universität Leipzig zur Sicherung guter wissenschaftlicher Praxis kenne und eingehalten habe.

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Datum Unterschrift

Darstellung des eigenen Beitrags

Publikation Nr.1

Proof of own contribution

Rockstroh A, Barzon L, Pacenti M, Palù G, Niedrig M, Ulbert S. Recombinant envelope-proteins with mutations in the conserved fusion loop allow specific serological diagnosis of dengue-infections. PLoS Negl Trop Dis. 2015 Nov 13;9(11):e0004218

The doctoral candidate Alexandra Rockstroh conceived and designed the study together with the corresponding author Sebastian Ulbert. She performed the majority of the experiments independently, e.g. design of the DNA constructs, cloning and transfection, establishment of stable *Drosophila S2* cells, protein expression and purification. She performed experiments to analyze the expressed mutant and wildtype DENV 1-4 E antigens in ELISAs with human polyclonal sera, which were provided by the co-authors Luiza Barzon, Monia Pacenti, Giorgio Palù and Matthias Niedrig. Alexandra Rockstroh analyzed, interpreted the data and designed the figures. The manuscript was drafted together with Sebastian Ulbert and discussed in conclusion with the co-authors.

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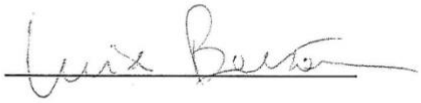

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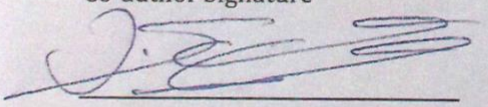

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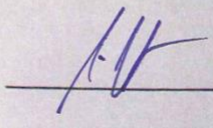

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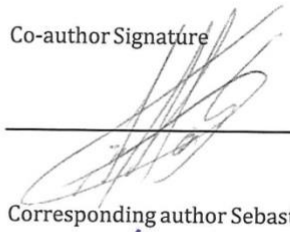

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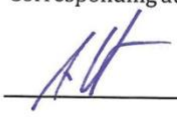

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Publikationsverzeichnis

Publikationen:

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Konferenzvorträge:

"Differentiation of flavivirus infections using a multi antigen ELISA based on recombinant envelope proteins with mutations in the conserved fusion loop domain"

→ 27th Annual Meeting of the Society for Virology, März 2017, Marburg

→ First International Conference on Zika Virus, Februar 2017, Washington D.C.

“Differentiation of dengue and Zika virus antibodies using envelope proteins with mutations in the conserved fusion loop domain”

→ Fraunhofer Life Science Symposium, November 2017, Leipzig

Patent:

Ulbert, S., Rockstroh, A., Distinguishing dengue virus infections from other flaviviral infections using a recombinant mutant envelope protein, EP3147294 A1, Fraunhofer-Gesellschaft zur Förderung der angewandten Forschung e.V,

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